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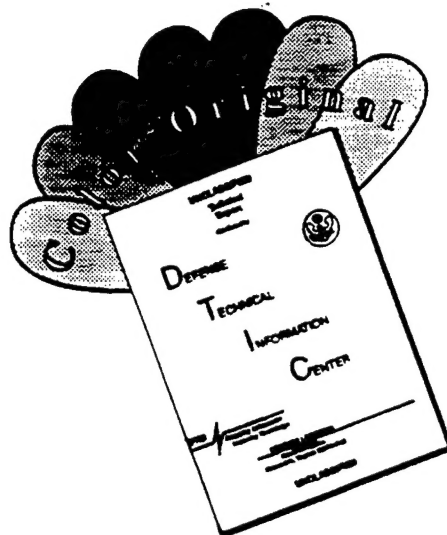
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## INTRODUCTION

### Background

Communication between individual cells and groups of cells is an integral part of development and differentiation. Signals from neighboring cells often activate key cell fate decisions. In many instances, the same signaling molecules that affect cell lineage differentiation and pattern formation also control cell proliferation. Two particularly intriguing examples of this are the proteins encoded by the *Drosophila* gene *wingless* (*wg*) and its mammalian homologue *Wnt-1* (1,2). These proteins share 64% sequence identity and may be functionally equivalent (3). Both contain a hydrophobic signal peptide; multiple, conserved cysteine residues; N-linked glycosylation site(s); and no discernible transmembrane domain (3). Null mutations in *wg* and *Wnt-1* are embryonic lethal, causing severe congenital malformation of the developing epidermis and central nervous system (4,5). *wg* and *Wnt-1* also regulate cell growth in certain cell types. *wg* is required for normal cell proliferation in the Malpighian tubule anlage (6), the developing wing discs (7), and neuroblasts (8); while ectopic expression of *Wnt-1* can stimulate cell division in the CNS (9) and induce growth of mammary tumors in adult mice (2).

It has been postulated that *wg* and *Wnt-1* are extracellular signaling proteins which mediate intercellular communication, thereby regulating differentiation. However, very little is known about the biochemical nature of this cell-cell signaling. That is, how is the *wingless* protein (WG) synthesized and secreted? How is WG transmitted from cell to cell? How is it received? And how are different cells able to respond in a position-specific manner?

Other genes in the *wg* / *Wnt-1* pathway.

Several other genes in the pathway have been identified genetically (for recent review see 10-12). A working model of how these genes interact is shown in Figure 1. Much of the *wg* signal transduction pathway is highly conserved throughout evolution. *armadillo* (*arm*), *dishevelled*, and *zeste white(3)* encode proteins which have known mammalian homologues. *arm* is the best characterized of the three; it encodes the *Drosophila* homologue of the vertebrate protein  $\beta$ -catenin. In *Drosophila*, cells respond to the *wg* signal by increasing the level of ARM. In vertebrates, cells respond by regulating  $\beta$ -catenin (13). Recent evidence indicates that ARM/ $\beta$ -catenin also interacts with the APC protein encoded by the tumor suppressor *adenomatous polyposis coli* gene. (14).

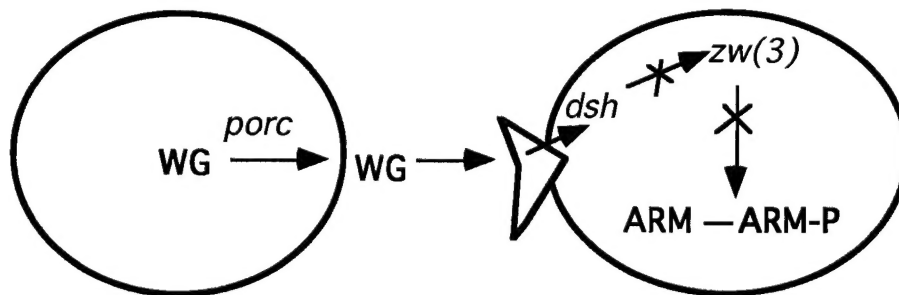


Figure 1. The *wg* / *Wnt-1* Signal Transduction Pathway.

The cell on the left expresses and secretes WG. *porcupine* (*porc*) is required for secretion of WG. *dishevelled* (*dsh*) is the first gene known to act after reception of the WG signal. *dsh* represses *zeste white (3)* kinase (*zw(3)*) activity and there is a concomitant increase in the amount of unphosphorylated ARM protein present (15).

A definitive receptor for WG has not been identified yet. However, recent work indicates that the *frizzled* gene family may encode WNT receptors (16). The *frizzled* genes encode novel receptor-like proteins with seven-pass transmembrane domains. Two *frizzled* genes have been identified in *Drosophila* (*fz-1* and *fz-2*). Both are candidate receptors for WG. Both bind to WG, although nothing is yet known about the affinity of these interactions. Transgenic S2 cells expressing either *fz-1* or *fz-2* on their surface will respond to WG, while control S2 cells will not. These observations raise several questions about potential receptor ligand interactions in vivo. Can WG bind to both receptors? Do the *fz-1* and *fz-2* receptors induce similar downstream responses?

### **Purpose of Present Work**

Genetic and molecular studies have shown that *wg* and its murine counterpart, the proto-oncogene *Wnt-1*, encode secreted proteins. These proteins are thought to act as extracellular signals that control growth and cell fate decisions in neighboring cells. Our goal is to understand how the signal is transmitted from cell to cell, and how the information is transduced in the receiving cells. The work outlined in this grant has divided into three sections:

**First:** We have constructed a tissue culture line which secretes soluble, active WG protein. WG activity can be followed using a second responding cell line, clone-8 cells. When clone-8 cells are grown in the presence of active WG protein, they express high levels of ARM protein. With both a source of soluble WG protein, and an assay for activity, we will purify WG protein, and determine if WG alone constitutes the signal or if other components are also required. **Second:** Identify and analyze accessory proteins that associate specifically with WG on the surface of the signaling cells and the responding cells, including candidate receptor molecules. **Third:** Secreted WG protein regulates the expression, and perhaps the cytoplasmic location of ARM. We will define when during embryogenesis *wg* activity is needed for proper *armadillo* expression. Specifically, is *wg* required for maintenance as well as initiation of ARM accumulation?

## BODY / PROGRESS

During the past year we have authored two manuscripts describing results from work outlined in this grant. One of the papers, "Glycosaminoglycans Can Modulate Extracellular Localization of the *wingless* Protein and Promote Signal Transduction" 1996, is in press in the *Journal of Cell Biology*. The other, "Post-Translational Modification and Secretion of *wingless* Protein" has been submitted to *Current Biology* (9/13/96). I have summarized both papers and included a brief description of related studies in this section of the Progress Report. Copies of the papers have been included in the Appendix.

### Specific Aim 1: Purification of and Characterization of WG Protein.

Our goal is to understand the extracellular steps in the *wg* / *Wnt-1* signaling pathway. In the past, biochemical analyses of *wg* / *Wnt-1* function have lagged behind genetic studies due primarily to the lack of an in vitro assay and purified WG protein. Recently, two in vitro assays have been developed for WG. In order to obtain WG protein in quantities sufficient for purification, we have constructed a genetically engineered cell line (S2HSWG(+)) which secretes active, affinity tagged WG (17). We have measured WG activity in both assays. In the first assay, S2HSWG(+) cells are co-cultured with specific populations of embryonic cells purified from viable *Drosophila* embryos by Whole Animal Cell Sorting (WACS) (17,18). The embryonic cells respond to WG expression by expressing specific downstream genes, such as *engrailed* (17). In the second assay (19), clone-8 cells, which are an established cell line derived from wing discs, are treated with conditioned media from S2HSWG(+) cells. In response to the WG signal, the clone-8 cells express higher levels of unphosphorylated ARM. Both the level of ARM expression, and the ratio of phosphorylated to unphosphorylated ARM is regulated by WG protein. Using S2HSWG(+) cells as a source of WG, and the clone-8 cell activity assay, we are characterizing the expression and secretion of WG as well as purifying the active WG protein.

### I. Post-Translational Modification, and Secretion of WG.

This work is described in a paper entitled "Post-Translational Modification and Secretion of *wingless* Protein" Laurie Smith, Xiwei Wang, and Susan Cumberledge. Submitted to *Current Biology*

Summary: The *Drosophila* gene *wingless* and its murine counterpart *Wnt-1* encode secreted proteins which act as short range signals during development. We have analyzed some of the steps required for the synthesis and secretion of active *wingless* protein (WG). Here, we report that *porcupine*, a gene thought to function upstream in the *wingless* pathway, is required for both modification and secretion of WG. We show that three distinct electrophoretic forms of WG can be detected both in vivo and in transgenic S2 cells expressing *wingless*. Two of the isoforms contain N-linked high mannose oligosaccharides. In *porcupine*<sup>-</sup> animals WG glycosylation is inhibited, and there is an accumulation of the intermediate glycoform. By examining WG expression and secretion in specific neuronal cells in the optic lobes, we have found that WG is translocated along axon-like projections and secreted within the target regions of the developing ganglia of the optic lobe. In *porcupine*<sup>-</sup> animals, WG secretion is blocked very early. The protein accumulates in the cell bodies and is not translocated down the axons. Secretion of other growth factor-like proteins, such as *decapentaplegic* protein is not affected. Surprisingly, WG secretion can also be inhibited very late in the secretory pathway. In animals homozygous for a temperature sensitive allele of *wg*, *wg*<sup>IL114ts</sup>, the mutant protein is still translocated down the axons, but accumulates in the region near the cell membrane. Our results demonstrate that WG is post-

translationally modified by the addition of high mannose glycans. Loss of *porcupine* activity disrupts both post-translational modification and translocation of WG. Our findings that WG secretion can be blocked both early and late in the secretory pathway, without affecting secretion of other proteins, lead us to propose that WG secretion may be regulated rather than constitutive.

## II. Protein Purification.

We have made good progress purifying active, secreted WG from S2HSWG(+) cells. Briefly, conditioned medium containing soluble WG is harvested from S2HSWG(+) cells. The soluble WG is bound to heparin agarose beads. Weakly binding proteins are washed from the column, and WG is eluted in the .75M to 1M NaCl wash. We estimate that these steps results in a 50% yield of WG protein, and a 200 fold purification. It is also a quick effective way to process large volumes of media, and to concentrate the WG protein. We have found that the amount of WG present in the medium varies depending on the precise growth conditions used. Under optimal conditions, WG is approximately 0.01% of the starting material. One particularly interesting finding to come from these purification studies is that the activity of partially purified WG is dramatically stimulated by the addition of exogenous glycosaminoglycans (see below for discussion). We are now testing a variety of ion exchange and gel filtration columns in order to chose an effective next step. As we continue to purify WG we will learn whether glycosaminoglycans are absolutely required for WG activity.

## III. Construction of an affinity tagged form of WG.

In earlier work, we had constructed and tested two affinity tagged forms of WG. While both forms were expressed in transgenic S2 cells, neither form was secreted into the soluble medium, making them of little use for protein purification. Because of the power of this technique, and the fact that it has been used successfully to purify other secreted proteins, we have constructed a third affinity tagged form of WG. This construct carries a HIS tag linked to a spacer arm at the carboxyl terminus of the protein. We are currently subcloning the WG-HIS cDNA into an appropriate expression vector. We will express the tagged WG in S2 cells and test for secretion and activity.

## Specific Aim 2: Identification of cell surface accessory proteins and candidate receptor molecules.

### I. Identification of cell surface accessory proteins.

This work is described in the paper "Glycosaminoglycans Can Modulate Extracellular Localization of the *wingless* Protein and Promote Signal Transduction" Frieda Reichsman, Laurie Smith, and Susan Cumberledge 1996, In Press, *Journal of Cell Biology*.

Summary: *wingless*, the Drosophila homologue of the proto-oncogene *Wnt-1*, encodes a secreted glycoprotein which regulates differentiation and proliferation of nearby cells. Here we report on the biochemical mechanism(s) by which the *wingless* signal is transmitted from cell to cell. When expressed in S2 cells, the majority (~83%) of secreted *wingless* protein (WG) is bound to the cell surface and extracellular matrix through specific, non-covalent interactions. The tethered WG can be released by addition of exogenous heparan sulfate and chondroitin sulfate glycosaminoglycans. WG also binds directly to heparin agarose beads with high affinity. These data suggest that WG can bind to the cell surface via naturally

occurring sulfated proteoglycans. Two lines of evidence indicate that extracellular glycosaminoglycans on the receiving cells also play a functional role in WG signaling. First, treatment of WG-responsive cells with glycosaminoglycan lyases reduced WG activity by 50%. Second, when WG-responsive cells were pre-incubated with 1 mM chlorate, which blocks sulfation, WG activity was inhibited to near-basal levels. Addition of exogenous heparin to the chlorate-treated cells was able to restore WG activity. Based on these results, we propose that WG belongs to the group of growth factor ligands whose actions are mediated by extracellular proteoglycan molecules.

## **II. Evaluation of FZ-2 as a candidate WG receptor.**

We have recently begun constructing stable transgenic S2 cells expressing *fz-2* protein (FZ-2). We have transfected S2 cells with a pMK *fz-2* expression vector (provided by J. Nathans) and are currently selecting stable transformants. Once we have obtained relatively pure WG protein, we will begin carrying out ligand receptor binding studies. These experiments will provide valuable information about the affinity of WG-FZ-2 interactions. In the upcoming year we will also carry out similar studies with FZ-1.

## **CONCLUSIONS**

During year 2 of the grant we have made significant progress in two of our specific aims. We have shown that post-translational modification and translocation of WG depends on *porcupine* activity and that WG may be secreted via a specialized pathway. We also have accumulated several lines of evidence that glycosaminoglycans act as accessory factors, promoting WG activity. Recently, it has been hypothesized that the *frizzled*-like genes may encode WNT receptors. In the next two years we will examine the interactions between WG and the *Drosophila* FZ-1 and FZ-2 receptor-like proteins.

These studies are helping to create a picture of how the WG/Wnt-1 signal is transmitted. Understanding the extracellular events involved in signal transduction is an integral part of understanding the etiology of cancer. Because these events are extracellular they are particularly attractive candidates for future work focusing on modification of the signaling response, and treatment of breast cancer.

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Glycosaminoglycans Can Modulate Extracellular Localization of the *wingless* Protein and Promote Signal Transduction

Running Title: Glycosaminoglycans Modulate *wingless* Signaling

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## Abstract

*wingless*, the *Drosophila* homologue of the proto-oncogene *Wnt-1*, encodes a secreted glycoprotein which regulates differentiation and proliferation of nearby cells. Here we report on the biochemical mechanism(s) by which the *wingless* signal is transmitted from cell to cell. When expressed in S2 cells, the majority (~83%) of secreted *wingless* protein (WG) is bound to the cell surface and extracellular matrix through specific, non-covalent interactions. The tethered WG can be released by addition of exogenous heparan sulfate and chondroitin sulfate glycosaminoglycans. WG also binds directly to heparin agarose beads with high affinity. These data suggest that WG can bind to the cell surface via naturally occurring sulfated proteoglycans. Two lines of evidence indicate that extracellular glycosaminoglycans on the receiving cells also play a functional role in WG signaling. First, treatment of WG-responsive cells with glycosaminoglycan lyases reduced WG activity by 50%. Second, when WG-responsive cells were pre-incubated with 1 mM chlorate, which blocks sulfation, WG activity was inhibited to near-basal levels. Addition of exogenous heparin to the chlorate-treated cells was able to restore WG activity. Based on these results, we propose that WG belongs to the group of growth factor ligands whose actions are mediated by extracellular proteoglycan molecules.

## Non-standard abbreviations:

ARM: *armadillo* protein; ECM: extracellular matrix; FGF: fibroblast growth factor; GAG: glycosaminoglycan; HRP: horseradish peroxidase; S2HSWG(+): *Drosophila* Schneider Line 2 (S2) cells transformed with a *wg* cDNA minigene; S2HSWG(-): *Drosophila* Schneider Line 2 (S2) cells transformed with a *wg* cDNA minigene in the antisense orientation; SO<sub>4</sub>-GAG: sulfated glycosaminoglycan; *wg*: *wingless* gene; WG: *wingless* protein.

## Introduction

Communication between cells is an integral part of development and differentiation. Cells determine their fates, in part, by where they are located relative to other cells. Work in *Drosophila* has shown that this positional information is often provided by the distribution of specific extracellular ligands such as the *wingless*, *hedgehog*, and *decapentaplegic* proteins (for recent reviews, see Klingensmith and Nusse, 1994 and Siegfried and Perrimon, 1994). Anterior/posterior, dorsal/ventral, and proximal/distal axes provide the framework for subsequent development of many tissue and organs. Loss of *wingless* activity alters many of these positional axes, severely disrupting epidermal patterning, appendage formation, and CNS development in *Drosophila* (Siegfried & Perrimon, 1994).

Similar signaling systems are also present in vertebrates. For example, *Wnt-1* is required for fetal brain development in mice (McMahon & Bradley, 1990), and body axis specification in *Xenopus* (McMahon & Moon, 1989). Several lines of evidence indicate that WG and Wnt-1 are both structural and functional homologues (Nusse & Varmus, 1992). The two proteins share 54% amino acid sequence identity; both are glycosylated, cysteine-rich and have a hydrophobic signal sequence. Both WG and Wnt-1 can regulate proliferation of specific neuronal cells during CNS development (Dickinson et al., 1994; Kaphingst & Kunes, 1994). *wg* can even substitute for *Wnt-1* in some functional assays in vitro; for example, RAC mammary epithelial cells can be transformed via expression of either Wnt-1 protein or WG (Ramakrishna & Brown, 1993).

Much of the WG/Wnt-1 pathway downstream of signal reception has also been conserved across species. Genetic screens in *Drosophila* have revealed other genes in the *wg* pathway, such as *dishevelled*, *armadillo*, and *zeste-white 3*, which have vertebrate counterparts (Siegfried & Perrimon, 1994). The most striking example is the *armadillo* protein (ARM) which is 70% identical to vertebrate  $\beta$ -catenin (McCrea et al., 1991). Candidate receptor(s) for WG/Wnt-1 have been identified (Bhanot et al., 1996); and some of the downstream steps in the pathway have now been ordered. In response to the *wg* signal, intracellular *dishevelled* protein

inhibits the activity of the *zeste-white 3* protein kinase (ZW3) (Siegfried et al., 1992). Inhibition of ZW3 allows activation of ARM/ $\beta$ -catenin, prompting an increase in the dephosphorylation of ARM and an increase in the amount of cytoplasmic ARM (Peifer et al., 1994a and b). These changes in the state of ARM ultimately result in regulation of gene expression, although the mechanism by which the signal is transmitted to the nucleus is not known.

In vitro experiments have shown that WG/Wnt-1 modulation of ARM/ $\beta$ -catenin activity can also affect cadherin activity, cell adhesion, cell movement, gap junctions, and perhaps the function of adherens junctions in some cell types (Moon et al., 1993a and b; Peifer, 1995). Recently, Hartenstein et al. (1994) have proposed that the delamination and division of certain *Drosophila* neuroblasts can be triggered by cytoskeletal changes which occur in response to the WG signal. It is not known whether WG can regulate cytoskeletal structure simply by modulating ARM and cadherin activity, or if these effects are more indirect. The *wg/Wnt-1* pathways may also interact with other intercellular signaling systems, including the *Notch* (Couso & Martínez Arias, 1994), *noggin* and *follistatin* (McGrew et al., 1995), *FGF* (e.g., Pan et al., 1995; Parr and McMahon, 1995) and *decapentaplegic/TGF $\beta$*  pathways (Pankratz & Hoch, 1995). Such interactions could occur downstream of signal reception, or extracellularly at the level of ligand and receptor interactions.

Significant progress has been achieved in our understanding of the intracellular steps involved after cells receive the signal, but what are the extracellular events leading to signal transduction? WG and Wnt-1 are much larger (468 and 370 amino acids, respectively) than most growth factors. How these ligands are transmitted from cell to cell and the molecules that govern their extracellular localization are not known. During early embryogenesis, extracellular WG is restricted to within one or two cell diameters of the WG-secreting cells in the epidermis, and the protein is distributed symmetrically. But later, during germ band retraction, the distribution becomes quite asymmetrical (Bejsovec & Martínez Arias, 1991). At that time WG is found several cell diameters anterior to the secreting cells, but is not detected on the posterior

side. These observations indicate that localization of extracellular WG may be regulated in a dynamic fashion.

Work in vitro (Bradley and Brown, 1995) suggests that Wnt-1 activity may require an accessory molecule which is secreted by C57MG epithelial cells, but not by Rat-2 fibroblast cells. Extracellular WG and Wnt-1 are both associated tightly with the cell surface (Papkoff & Schryver, 1990; van den Heuvel et al., 1993; Smith et al., submitted) and extracellular matrix (Bradley & Brown, 1990; Gonzalez et al., 1991; van den Heuvel et al., 1993; Smith et al., submitted). The nature of these interactions is not known. However, Wnt-1 can be released from the cell surface by treatment with exogenous heparin (Bradley & Brown, 1990), suggesting that Wnt-1 protein may interact with cell surface associated proteoglycans. Proteoglycans are a distinct class of glycoproteins that contain covalently linked glycosaminoglycans (GAGs) such as heparan sulfate and chondroitin sulfate (Kjéllan & Lindahl, 1991). They can bind to a variety of growth factors, and in some cases function as co-receptors (Klagsbrun & Baird, 1991; Schlessinger et al., 1995). For example, secreted bFGF associates with the cell surface proteoglycan syndecan-1 (Bernfield & Hooper, 1991). Both syndecan-1 and the FGF receptor are required for transmembrane signaling (Yayon et al., 1991). Similarly, betaglycan binds to TGF $\beta$  and promotes TGF $\beta$  binding to its high affinity receptor (López-Casillas et al., 1993).

We are interested in the mechanism by which extracellular WG becomes localized, how the ligand travels from cell to cell, and how the WG signal is transmitted across the membrane. Here, we show that secreted WG can bind to glycosaminoglycans with high affinity. These interactions are specific and are mediated, in part, by carbohydrate moieties found on the WG protein. Extracellular diffusion of WG in vivo may be restricted by binding to cell surface proteoglycans. Furthermore, we show that interactions with glycosaminoglycans can promote WG signal transduction. Proteoglycans are known to function as co-receptors for certain vertebrate growth factors; we propose that transmission of the WG signal may occur via a similar mechanism.

## Materials and Methods

### Cell Culture

The WG-expressing cell line S2HSWG(+) contains the *wg* cDNA under the control of the heat shock promoter (Cumberledge & Krasnow, 1993). S2HSWG(-) cells are identical except that the *wg* cDNA is in the anti-sense configuration. S2 cells were cultured in Schneider's Drosophila Medium (Gibco) supplemented with 10% fetal calf serum (Gibco) and penicillin/streptomycin (Gibco, 25 units/ml and 25 µg/ml respectively) at 24°C. Clone-8 cells were grown in Shields and Sang M3 Insect Medium (Sigma) supplemented with insulin (Sigma, 0.125 IU/ml), 2% fetal calf serum (Gibco), penicillin/streptomycin (Gibco, as above), and 2.5% fly extract (Currie et al., 1988).

### Preparation of Conditioned Medium

S2HSWG(+) cells were grown for 24-48 h, to a density of  $6-8 \times 10^6$  cells/ml in flasks or  $10-12 \times 10^6$  cells/ml in suspension. The cells were then heat shocked for 45 min-1 h at 37°C, washed twice, and allowed to recover at  $20 \times 10^6$  cells/ml in serum-free M3 medium at 24°C for 2-3 h. The conditioned medium was separated from cells by centrifugation at 2,000g for 5 min and then cleared by centrifugation at 20,000g for 15 min at 4°C. In control experiments when cells were pulsed with [<sup>35</sup>S] Met, there was a 1 h lag after heat shock before soluble [<sup>35</sup>S]-WG could be detected in the medium (not shown).

For release of WG from the cell surface using GAGs, 10 µg/ml of heparin (Sigma; MW 6,000), heparan sulfate (Sigma; MW 7,500) or chondroitin sulfate (Sigma; contains 70% chondroitin sulfate A and 30% chondroitin sulfate C, which differ solely in the position of the single sulfate per disaccharide) was added to S2HSWG(+) cells following heat shock and harvest, at the beginning of the two hour recovery period. Conditioned media were concentrated in Centriprep-30 concentrators (Amicon) and media from equal numbers of cells were resolved by SDS-PAGE and transferred to nitrocellulose. Ponceau S protein stain (Sigma) was used to verify equal loading of total protein in each lane. Blots were probed with

polyclonal rabbit  $\alpha$ -WG and goat  $\alpha$ -rabbit HRP antibodies (Bio-Rad), and protein bands were visualized using Enhanced Chemiluminescence (Amersham) or Super Signal (Pierce). For concentration dependence experiments, conditioned medium was diluted with M3 medium to the specified concentrations and applied to clone-8 cells as described below. No WG was detected in Western analyses of conditioned media from S2HSWG(-) cells (not shown).

### **Preparation of F(ab)<sub>2</sub> fragment for immunoprecipitation**

To differentiate between antibody and WG antigen during immunoprecipitation experiments (WG and the IgG heavy chain have very similar apparent molecular weights, 55 kDa), we used biotinylated rabbit  $\alpha$ -WG F(ab)<sub>2</sub> fragment (25 kDa) for all immunoprecipitations. F(ab)<sub>2</sub> fragment was generated from whole rabbit  $\alpha$ -WG antibody by digesting with 20  $\mu$ g pepsin (Sigma) per mg of antibody in 100 mM sodium citrate, pH 3.5, for 6 h at 37°C. The reaction was stopped with 1/10 volume of 3.0 M Tris, pH 8.0, and the sample was dialyzed against 0.1 M sodium borate, pH 8.8. To biotinylate the F(ab)<sub>2</sub>, 250  $\mu$ g N-hydroxysuccinimide ester (10 mg/ml in dimethyl sulfoxide) was added per mg of antibody and the solution was incubated at room temperature for 4 h. The reaction was stopped by adding 20  $\mu$ l 1M NH<sub>4</sub>Cl per 250  $\mu$ g ester and incubating at room temperature for 10 min. Free biotin was removed by exhaustive dialysis against phosphate buffered saline (PBS). Avidin agarose beads (Pierce) were used to immunoprecipitate the F(ab)<sub>2</sub>-protein complexes. The efficiency of immunoprecipitation from the medium was measured by quantitative Westerns (see below). Greater than 85% of the soluble WG protein found in the starting material (conditioned medium) was recovered after immunoprecipitation (not shown).

### **Quantitation of WG protein**

The relative amounts of WG protein on the cell surface, medium, and ECM were measured by Western blotting using rabbit  $\alpha$ -WG primary antibody followed by [<sup>125</sup>I] goat  $\alpha$  rabbit antibody (ICN) and phosphorimager analysis. S2HSWG(+) cells (8 x 10<sup>6</sup> cells /ml) were heat

shocked and allowed to recover for 2 h, then the whole cells, medium, and ECM were separated. For immunoprecipitation from the cell surface, cells were washed and resuspended in M-3 medium + 1% BSA.  $\alpha$ -WG F(ab)<sub>2</sub> (1:500 dilution) was added and incubated with gentle mixing for 45 min at 4°C. Cells were harvested by centrifugation, washed 2X in PBS and lysed in 1 ml RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) by incubating for 15 min on ice. The lysate was centrifuged at 14,000g to remove particulate matter. Avidin conjugated agarose beads (Sigma) pre-absorbed with 1% BSA were added to the supernatant and incubated for 1 h at 4°C with gentle mixing. Beads were then sedimented at 14,000g for 15 s, washed 3 times in RIPA buffer, resuspended in SDS sample buffer (50 mM Tris, 2% SDS, 10% glycerol) and boiled 5 min. For immunoprecipitation from the medium,  $\alpha$ -WG F(ab)<sub>2</sub> antibody was added to the medium (1:500), and the mixture was incubated with gentle mixing for 45 min at 4°C. Avidin-agarose beads were then added and incubated for 1 h at 4°C with gentle mixing. Beads were sedimented at 14,000g for 15 s, washed 3 times in RIPA, resuspended in SDS sample buffer and boiled 5 min. The ECM fraction was prepared after the cells were removed. First, the flask was washed 5 times with PBS, incubated for 5 min with 4 ml of RIPA, then washed 5 times with RIPA buffer. The ECM was then extracted from the flask by adding 2X SDS sample buffer, boiling for two minutes, and scraping with a cell scraper. Samples were electrophoreses on a 10% SDS gel and transferred to nitrocellulose (Schleicher & Schuell). The filter was blocked with 3% milk PBST (PBS, 0.05% Tween 20) probed with rabbit  $\alpha$ -WG antibody (1:15,000) for 1 hr, washed and then probed with [<sup>125</sup>I]-goat  $\alpha$  rabbit antibody (1 $\mu$ Ci/ml) for 2 h. The nitrocellulose was washed and then exposed to a Phosphor Screen. The screen was subsequently scanned on a Storm Phosphorimager (Molecular Dynamics), the image analyzed, and the amount of WG protein in each lane quantitated using Imagequant software (Molecular Dynamics).

The absolute amount of WG in the medium was measured by metabolic labeling of cells with [<sup>35</sup>S]-Methionine followed by immunoprecipitation of WG from the medium. Log phase

S2HSWG(+) cells were washed and then resuspended ( $8 \times 10^6$  cells/ml) in M3 medium without methionine for 30 min. [ $^{35}\text{S}$ ]-Methionine was added at 350  $\mu\text{Ci}/\text{ml}$  for 3 h including heat shock and recovery. For immunoprecipitation from the medium, whole rabbit  $\alpha$ -WG antibody was added and incubated with gentle mixing for 45 min at  $4^\circ\text{C}$ . Protein A beads were then added and incubated for 1 h at  $4^\circ\text{C}$  with gentle mixing. Beads were sedimented at 14,000g for 15 s, washed 3 times in RIPA, resuspended in SDS sample buffer and boiled 5 min. The sample was electrophoresed on a 10% SDS gel. For fluorography, the gel was treated with Entensify according to the manufacturer's instructions (DuPont), then dried and exposed to film. After developing, the WG bands were excised and quantitated by scintillation counting. The efficiency of [ $^{35}\text{S}$ ]-Met incorporation, or specific activity of each preparation, was determined by measuring the total amount of radioactivity incorporated (quantitated as the TCA precipitable counts), and the protein concentration determined by BCA assay (Sigma). The amount of WG protein in the medium was calculated by dividing the cpm incorporated (in the WG band) by the specific activity of the total protein. This number estimates the amount of WG present, but is influenced by the number of Met residues in WG compared to the average number of Met residues in the total protein preparation. A typical specific activity of the total protein was  $1.6 \times 10^4$  cpm/ $\mu\text{g}$  protein.

### **Heparin-Agarose Affinity Chromatography**

Conditioned medium from S2HSWG(+) cells was gently mixed with heparin-4% agarose beads (Sigma) overnight at  $4^\circ\text{C}$ . Beads were collected by centrifugation at 2,000g for 2 min, washed with 20 volumes of 50 mM NaCl/10 mM HEPES, and eluted at room temperature for 5-15 min in the specified concentrations of NaCl/10 mM HEPES.

### **Activity Assays**

Clone-8 cells were plated into 6-well plates (Corning and Falcon), grown to 70-90% confluency, and then incubated with conditioned media from S2HSWG(+) cells for 2.5-3 h.



Following WG or control treatment, medium was removed by aspiration and the clone-8 cells were harvested in PBS and lysed in lysis buffer (50 mM Tris, 2% SDS). The total protein concentration was measured by BCA assay (Sigma) and equal amounts of protein were loaded in each lane of an SDS polyacrylamide gel and resolved by SDS-PAGE. The proteins were transferred to nitrocellulose or PVDF membrane and stained with Ponceau S (Sigma) to verify equal loading of each lane on the gel. Blots were then probed with mouse monoclonal  $\alpha$ -ARM antibody N27A1 and goat  $\alpha$ -mouse-HRP (Bio-Rad). Immunoreactive protein bands were visualized by treating the blots with Enhanced Chemiluminescence (Amersham) or Super Signal (Pierce) as per the manufacturer's instructions and then exposing the blot to X-ray film. The film was analyzed by densitometry scanning and the amount of ARM was quantitated using NIH Image software. WG activity was quantified as the increase in dephosphorylated ARM. As a third, independent control for equal protein loading, some blots were also probed with  $\alpha$ -HSP70 antibody. Data normalized using the HSP70 loading controls were not significantly different from non-normalized data. No change in ARM amount or distribution was detected in assays of conditioned media from S2HSWG(-) cells (not shown).

For enzyme treatments, clone-8 cells were incubated with heparin lyases I (heparinase) and III (heparatinase) and chondroitin sulfate ABC lyase (Seikagaku America) at 1.0 mIU/ml overnight prior to and during WG treatment. Conditioned medium from S2HSWG(+) cells was treated with the same concentration of the lyases for 30 min immediately prior to use in the assay. For sodium perchlorate treatment, clone-8 cells were incubated with 1 mM sodium perchlorate (final concentration) 48 h prior to as well as during the incubation with WG in conditioned medium. Heparin and chondroitin sulfate (see Preparation of Conditioned Medium, above) were added at 10  $\mu$ g/ml where indicated.

## RESULTS

### Extracellular Localization of WG

*Drosophila* Schneider Line 2 (S2) cells transformed with a *wg* cDNA minigene (S2HSWG(+)) secrete active *wingless* protein (WG) (Cumberledge & Krasnow, 1993; van Leeuwen et al., 1994). Figure 1 (lanes 1 and 2) shows that three distinct electrophoretic forms of WG with apparent molecular weights of 52, 55, and 57 kDa can be immunoprecipitated from S2HSWG(+) whole cell lysates; no WG is detected in control cells. The two larger species (forms II and III) are N-linked glycoproteins, and the fastest migrating form is unglycosylated (form I) (Smith et al., submitted). van den Heuvel, et al. (1993) have shown previously that one WG species can be found in the medium, on the cell surface and on the extracellular matrix (ECM). By comparison, when Wnt-1 is expressed in various cell lines, the secreted protein is usually restricted to the cell surface and ECM. Of the cell types that have been examined thus far, only C57MG/Wnt-1 cells secrete soluble Wnt-1 into the medium (Bradley & Brown, 1990; Papkoff & Schryver, 1990; Bradley and Brown, 1995). Multiple glycoforms of Wnt-1 have also been detected and different transgenic cell lines express different forms. These observations raise several questions: are all three forms of WG secreted, where is the majority of the extracellular WG, and does glycosylation affect the extracellular localization of WG?

Figure 1a (lanes 3 and 4) shows that all three WG isoforms are also present on the cell surface and in the medium. Both the glycosylated and the unglycosylated forms are secreted. Furthermore, the relative abundance of each form does not vary significantly between locations: most of the protein is found as form III, with only a small amount of form I present. Multiple WG isoforms can also be detected in the ECM fraction (Figure 1a lane 5). These isoforms resolve poorly on SDS polyacrylamide gels, making it difficult to identify unequivocally which glycoforms are present. We do not know if the aberrant behavior reflects a change in the composition of the WG protein, or if it is an artifact of the method used to prepare the ECM fraction. We also quantitated the relative amount of WG

protein present in each extracellular location (Figure 1b). Of the total extracellular WG, 50% was localized to the cell surface, 17% to the medium, and 33% was found associated with the ECM. We conclude that the majority of extracellular WG is tethered to the cell membrane and ECM and a small fraction is soluble in the medium.

Next, we examined how WG is bound to the cell surface. We tested several factors for their ability to release WG. When S2HSWG(+) cells were treated with 0.5 M NaCl or 0.005% triton X-100, no WG was released from the cell surface (data not shown). Previous work by Bradley and Brown (1990) has shown that addition of exogenous heparin, an abundant and naturally occurring sulfated glycosaminoglycan (SO<sub>4</sub>-GAG) can release Wnt-1 from the cell surface. Therefore, we treated heat-shocked S2HSWG(+) cells with medium plus exogenous SO<sub>4</sub>-GAGs. In contrast to NaCl treatment, addition of as little as 10 µg/ml heparin, heparan sulfate, or chondroitin sulfate releases bound WG from the cell surface (Figure 2). After a 2 hour incubation in the presence of SO<sub>4</sub>-GAGs, there is a 5-10-fold increase in soluble WG in the medium as compared to the control treatment. This competition is quite specific and cannot be accounted for simply by differences in charge density, as heparin and heparan sulfate preferentially release WG form III, whereas chondroitin sulfate releases WG form II. Thus WG binds to the cell surface through specific, non-covalent interactions.

### **WG is a Heparin Binding Protein**

Can SO<sub>4</sub>-GAGs mediate the release of WG by competing directly for binding sites on WG, or is the effect indirect? To address this question we tested whether WG can bind directly to heparin. When conditioned medium containing WG is incubated with heparin-agarose beads, WG binds to the beads quantitatively and with high affinity (Figure 3). WG is eluted from the column only after washing with 1.0 M NaCl. Form III appears to bind with somewhat stronger avidity to heparin agarose than either form I or II. This is consistent with our observation that heparin and heparan sulfate are more effective at releasing form III from the cell surface. Together, these results suggest that WG may associate with specific cell surface proteoglycans.

Is WG also bound to SO<sub>4</sub>-GAGs in the conditioned medium and on the ECM? Many proteoglycans found on the cell surface are shed into the medium, and would therefore be available for binding; however, we have not tested this directly. In the case of the ECM, preliminary results suggest that WG bound to the matrix can also be released by the addition of heparin and chondroitin sulfate (data not shown).

### **Sulfated Glycosaminoglycans Can Promote WG Signaling**

Before testing the role of glycosaminoglycans in WG signaling, we first examined normal WG activity using the ARM assay described by van Leeuwen et al. (1994). Previous genetic studies have shown that *zw(3)*, a ser/thr kinase, promotes the phosphorylation and inhibition of ARM protein. WG inhibits *zw(3)* activity, thereby activating ARM. When clone-8 cells, a *Drosophila* cell line derived from imaginal discs (Currie et al., 1988), are incubated with soluble WG in conditioned medium from S2HSWG(+) cells, there is a large increase in the ratio of dephosphorylated to phosphorylated ARM and a concomitant increase in the total amount of cellular ARM protein. We have quantitated WG activity in this assay by measuring the increase in the dephosphorylated (faster migrating) form of ARM. Figure 4 shows a concentration dependence curve for WG protein present in the conditioned medium of S2HSWG(+) cells. Using the ARM assay, we can detect WG activity at concentrations in the pM range (Figure 4). This range of activity suggests an interaction with a high affinity receptor.

We used two independent methods to test whether loss of specific SO<sub>4</sub>-GAGs would affect WG activity. First, we used glycosaminoglycan lyases to enzymatically remove SO<sub>4</sub>-GAGs from clone-8 cells and from S2HSWG(+) conditioned medium and then tested WG activity. Heparinase, heparatinase, and chondroitin ABC lyase cleave the SO<sub>4</sub>-GAGs heparin, heparan sulfate, and chondroitin sulfates, respectively, while leaving other carbohydrate moieties and sulfate groups intact (Linhardt, 1994). When clone-8 cells and conditioned medium were treated with these three SO<sub>4</sub>-GAG lyases there was a 50% loss of activity as compared to untreated control cells (Figure 4). No differences in proliferation, morphology, or

cell adhesion were noted between control and enzyme-treated clone-8 cells. Thus, enzymatic removal of extracellular  $\text{SO}_4$ -GAGs significantly impairs WG activity. Are the  $\text{SO}_4$ -GAGs supplied by the S2HSWG(+) conditioned medium or the clone-8 cells? Endogenous  $\text{SO}_4$ -GAGs can be found on the cell surface or be shed into the medium as proteoglycans. Preliminary studies treating only the clone-8 cells with the lyases yielded similar results, indicating that conditioned medium from S2HSWG(+) cells is probably not a sufficient source of  $\text{SO}_4$ -GAGs.

We also looked at the role of cell surface  $\text{SO}_4$ -GAGs using a second, independent approach. We inhibited sulfation of proteoglycans *in vivo* by treating cells with sodium perchlorate, a reversible, competitive inhibitor of ATP-sulfurylase (Farley et al., 1976; 1978). Rapraeger et al. (1991) have shown that using chlorate treatment to block sulfation of proteoglycans effectively blocks FGF-induced DNA synthesis and mitogenesis, as well as the binding of FGF to its high affinity receptor. If  $\text{SO}_4$ -GAGs promote WG activity, then chlorate treatment of the clone-8 cells should lower WG activity at submaximal WG concentrations. Figure 5 shows that after incubation with 1 mM sodium perchlorate, clone-8 cells are considerably less responsive to WG. In fact, at the submaximal concentrations of WG used, the response of the chlorate treated cells is inhibited to a near-baseline level. The chlorate inhibition can be abrogated by the addition of heparin. When chlorate-treated cells are exposed to WG in the presence of 10  $\mu\text{g/ml}$  heparin, they respond like untreated cells (Figure 5). Chondroitin sulfate is also able to restore activity. The effect of chlorate on the cells is not simply a generally debilitating one, since it is unlikely that the addition of GAGs would overcome an overall decline of cell function. Rather, the loss of WG activity in chlorate-treated cells is most likely due to the specific effect of blocking sulfation of GAGs on these cells, since supplying sulfated GAGs restores activity. Together, these results argue that sulfated GAGs can play an important role in promoting WG signaling.

Can the addition of exogenous  $\text{SO}_4$ -GAGs stimulate WG activity directly? In the case of FGF, addition of exogenous heparin can induce oligomerization of the ligand and promote FGF

receptor dimerization and signal transduction. In contrast, sucrose octasulfate, a persulfated disaccharide that binds FGF without ligand oligomerization, has no effect on FGF signaling (Spivak-Kroizman et al., 1994). We have found that supplementing WG conditioned medium with 10  $\mu\text{g/ml}$  heparin increases WG activity by as much as 80% (Figure 5). The addition of heparin alone has no effect on ARM, indicating that heparin activation is mediated through WG, and that WG and heparin act synergistically. Addition of chondroitin sulfate does not enhance WG activity (Figure 5). We did not test the effects of adding sucrose octasulfate, as it does not appear to bind WG (data not shown).

## **DISCUSSION**

### **Post-Translational Modification And Localization Of WG**

We find that ~17% of the extracellular WG secreted by S2HSWG(+) cells is in an active, soluble form. Characterization of WG expression in these cells suggests that glycosylation of WG is not required for secretion *in vitro*; nor does it affect the distribution of the protein to the cell surface, ECM, or medium. However, glycosylation may influence WG interactions with extracellular proteoglycans as discussed below. Once secreted, most of the extracellular WG is associated with the ECM and cell surface, probably via interactions with sulfated glycosaminoglycans. This tethering may account for the fact that *in vivo* extracellular WG protein can usually be detected only one to two cell diameters away from the secreting cells.

### **WG Interacts With Cell Surface Sulfated Glycosaminoglycans**

We have shown that WG can be released from the cell surface by addition of the  $\text{SO}_4$ -GAGs heparin, heparan sulfate, and chondroitin sulfate. In addition, WG binds to heparin-agarose beads with high affinity, suggesting that exogenous  $\text{SO}_4$ -GAGs probably promote WG release by direct competition for binding sites on WG. The interactions between WG and the  $\text{SO}_4$ -GAGs are specific: chondroitin sulfate preferentially releases WG form II, while

heparin and heparan sulfate release form III. In addition, form III binds to heparin agarose with a stronger affinity than form II, and neither form binds sucrose octasulfate. The biological effects of the SO<sub>4</sub>-GAGs are also specific: low concentrations of heparin stimulate WG activity, but chondroitin sulfate does not.

What factors might account for this specificity? The differences between these three GAGs are somewhat subtle. All are highly negatively charged, being composed of irregularly repeating disaccharide units that are N-acetylated, and N- and O-sulfated (Silbert et al., 1995). Heparin is the most highly sulfated of the three, averaging 2-2.5 sulfates per disaccharide vs. 1 for chondroitin sulfate and <1 for heparan sulfate (Silbert et al., 1995). Therefore it is unlikely that the degree of sulfation is responsible for the specificity of the interactions. The sugar makeup of the three GAGs correlates well with respect to their actions on WG. While all three contain N-acetyl-glucosamine, they differ in that heparin and heparan sulfate contain N-acetyl-iduronic acid whereas chondroitin sulfate contains N-acetyl-galactosamine (Silbert et al., 1995). This suggests that the hexosamine composition of the GAGs is an important binding determinant for specificity. Different glycosylation forms of WG could bind to different proteoglycans or to different GAGs on the same proteoglycan.

### **Sulfated Glycosaminoglycans Play a Functional Role in WG Signal Transduction In Vitro**

What is the biological significance of these interactions? Proteoglycans are expressed by most vertebrate cells and homologs have also been identified in *Drosophila* (Spring et al., 1994; Nakato et al., 1995). They often mediate cell-cell and cell-matrix interactions (Silbert et al., 1995). Some, such as syndecan-1 (Bernfield et al., 1993) and glypican (Vaughan et al., 1994), interact with growth factors (e.g., FGF, TGF $\beta$ ) during signal transmission (Silbert et al., 1995). Others, such as perlecan, bind to cell adhesion molecules (Hayashi et al., 1992).

Our results show that GAGs can also participate in WG signaling. The enzymatic digestion of SO<sub>4</sub>-GAGs in conditioned medium and on the clone-8 cell surface results in a

~50% loss of WG activity. The specificities of the lyases for the GAGs heparin, heparan sulfate, and chondroitin sulfates A and C suggest that the loss of activity is due directly to the loss of these GAGs. This idea is further collaborated by the finding that chlorate-treated cells respond poorly to WG. The loss of activity in response to chlorate treatment is most likely a specific result of decreased sulfation of GAGs, as addition of soluble chondroitin sulfate or heparin restores activity to the chlorate-treated cells. These results are also consistent with the notion that clone-8 cells, and not S2 cells, express the necessary GAGs. Only the clone-8 cells, and not the S2HSWG(+) conditioned medium, were subjected to chlorate treatment.

How might extracellular proteoglycans modulate WG activity? Of the growth factors that interact with cell surface proteoglycans, FGF is probably the best characterized. FGF requires both a "low affinity" syndecan receptor ( $K_d \sim 10^{-9}$  M), and a high affinity FGF receptor ( $K_d \sim 10^{-11}$  M) for mitogenic activity (Moscatelli, 1987; Klagsbrun & Baird, 1991). How does the low affinity receptor promote signaling if at equilibrium, the high affinity receptor will be saturated well before a significant fraction of the low affinity receptor is bound? Two models have been proposed to explain FGF signaling (see Klagsbrun and Baird, 1991 and Schlessinger et al., 1995, for discussion). Either of these can be adapted to WG signaling as shown in Figure 6. In the first model, WG binding to cell surface proteoglycans limits diffusion of the ligand to two dimensions. If the on/off rates of the low affinity receptors are high, the net effect will be to increase the local concentration of ligand available for binding to the high affinity receptors. We suggest that WG signaling may occur in an analogous manner. In the second model, ligand binding to the proteoglycan induces ligand oligomerization. This, in turn, promotes receptor clustering and transmembrane signaling. The data in this paper are consistent with either of these models. With the recent identification of *fz-2* as a potential WG receptor (Bhanot et al. 1996), it will now be possible to test these models using a more defined system.

Do proteoglycans interact with other WNT proteins? Wnt-1 binds to heparin agarose (Bradley & Brown, 1990), and recent work by Burrus and MacMahon (1995) has shown that



several other Wnt family members are released from the cell surface by the addition of exogenous heparin. Jue et al. (1992) have found that addition of 50-200  $\mu\text{g/ml}$  heparin can inhibit Wnt-1 transformation in a co-culture assay. Nevertheless, heparin effects on FGF activity have been shown to vary from enhancement to inhibition depending on experimental conditions and the concentrations of heparin tested (Schlessinger et al., 1995). It will be interesting to determine if lower concentrations of heparin can stimulate Wnt-1 activity. Our studies provide the first evidence that sulfated GAGs participate in WG signaling pathway. Given the remarkable degree of conservation between the WG and Wnt-1 signaling pathways we speculate that GAGs may also play a functional role in Wnt-1 activity.

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## FIGURE LEGENDS

### **Figure 1. Most Secreted WG Is Bound to the Cell Surface and Extracellular Matrix.**

(a) Left panel: Intracellular WG was immunoprecipitated from S2HSWG(+) whole cell extracts (lane 1) and S2HSWG(-) whole cell extracts (lane 2). The immunoprecipitates were analyzed using 10% SDS-PAGE and Western blotting techniques. Three WG isoforms are labeled. Right panel: Immunoprecipitated WG from the cell surface (lane 3) and medium (lane 4) and total ECM extract (lane 5) were analyzed using 10% SDS-PAGE and Western blotting techniques. The nitrocellulose blots were probed with rabbit  $\alpha$ -WG antibody followed by [ $^{125}$ I]-goat  $\alpha$  rabbit secondary antibody. Filters were analyzed using a Phosphorimager (Molecular Dynamics Storm Model 660). All immunoprecipitations were carried out with biotinylated rabbit  $\alpha$ -WG F(ab) $_2$  antibody. 1X is equivalent to material from  $10 \times 10^6$  cells. Control experiments show >85% of the soluble WG in the medium can be recovered after immunoprecipitation with  $\alpha$ -WG F(ab) $_2$  antibody and avidin-agarose beads (not shown).

(b) Relative distribution of extracellular WG. Data from (a) and two similar experiments were quantified using Imagequant (Molecular Probes).

### **Figure 2. Release of WG from the Cell Surface by Exogenous SO $_4$ -GAGs.**

Following heat shock, S2HSWG(+) cells were harvested into centrifuge tubes, washed, and resuspended in fresh, serum-free M3 medium. The cells were allowed to recover in the tubes for 2 h in the absence or presence of 10  $\mu$ g/ml heparin, chondroitin sulfate, or heparan sulfate. The conditioned media, containing the released WG, were concentrated, and material from  $7 \times 10^6$  cells was loaded in each lane of a 10% SDS polyacrylamide gel. Immunoblotting was performed as described in Figure 1.

### Figure 3 Heparin Affinity Chromatography.

Conditioned medium from S2HSWG(+) cells was incubated with heparin-4% agarose beads. The column was washed with 50 mM NaCl and fractions were eluted with 0.5, 1.0, 1.5, 2.0 and 4.0 M NaCl, followed by 2% SDS. Material from  $15 \times 10^6$  cells was loaded in each lane of an SDS gel, and then subjected to Western analysis as in Figure 1.

### Figure 4. Enzymatic Degradation of SO<sub>4</sub>-GAGs Inhibits WG Activity.

(a) Western analysis of the concentration dependence of WG activity in the absence or presence of SO<sub>4</sub>-GAG lyases. Control (untreated) and lyase-treated conditioned media from S2HSWG(+) cells were assayed for WG activity on control or lyase-treated clone-8 cells, respectively. 1X medium = approximately 4 nM WG, as measured by immunoprecipitation of [<sup>35</sup>S]-Met-labeled WG (see Methods). Lysates of the clone-8 cells were subjected to 8% SDS-PAGE (70 µg protein/lane) and Western analysis. The blots were probed with N27A1 mouse α-ARM and goat α-mouse-HRP antibodies, and immunoreactive proteins were visualized using enhanced chemiluminescence. In lighter exposures, the phosphorylated and dephosphorylated forms are clearly resolved as two separate bands in the lanes with stronger signals.

(b) Data from (a) and two similar experiments are represented graphically. The increase in the amount of dephosphorylated (faster migrating) ARM was used to quantitate WG activity in control (squares) vs. lyase-treated (triangles) clone-8 cells. Densitometry was performed using NIH Image. No ARM response was observed in clone-8 cells exposed to conditioned medium from S2 cells transfected with the *wg* cDNA mini-gene in the antisense orientation (S2HSWG(-) cells, not shown).

### Figure 5. Chlorate Treatment Blocks WG Activity.

Clone-8 cells grown in the absence or presence of 1 mM chlorate (as indicated) were mock treated (left panel) or treated with WG-conditioned medium from S2HSWG(+) cells (right panel). The concentrations of WG added (0.1-0.3X) were those that typically elicit ~50% of

WG activity at 1X (see fig. 3). Heparin and chondroitin sulfate were applied, where indicated, at 10  $\mu\text{g/ml}$ . The amount of dephosphorylated ARM in each lane of Western blots of the clone-8 cell lysates was quantified using NIH Image. All treatments are shown as a percentage of the control +WG value (100%). Bars indicate standard errors;  $n \geq 3$  for all treatments except chlorate + chondroitin sulfate in the absence of WG,  $n = 2$ .

**Figure 6. Models of WG Interaction with Cell Surface Accessory  
Proteoglycans and High Affinity Receptors.**

Two hypotheses for activation of WG signaling by  $\text{SO}_4$ -GAGs are illustrated. Model I: Localization of WG (blue) to the cell surface via  $\text{SO}_4$ -GAG chains (red) of a low affinity receptor (core protein in black) reduces the number of dimensions for diffusion of WG from three to two, increasing the chances that WG will interact with a high affinity receptor (green). Model II: Binding of WG to  $\text{SO}_4$ -GAG chains on a low affinity receptor on or shed from the cell surface induces oligomerization of WG and receptor clustering.

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Figure 1a

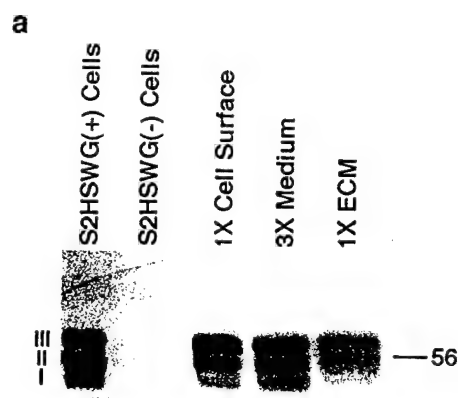


Figure 1b

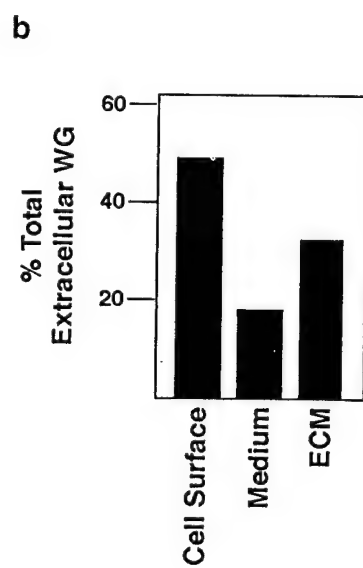


Figure 2

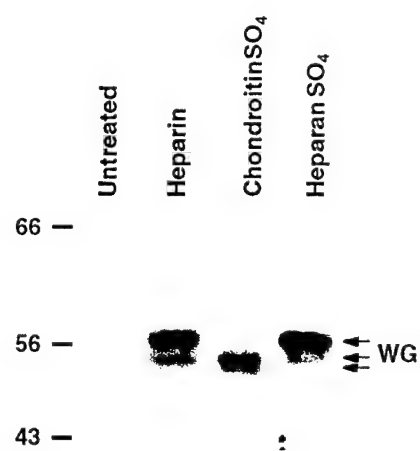


Figure 3

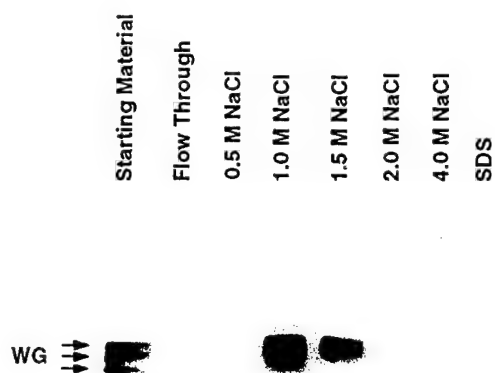


Figure 4 a

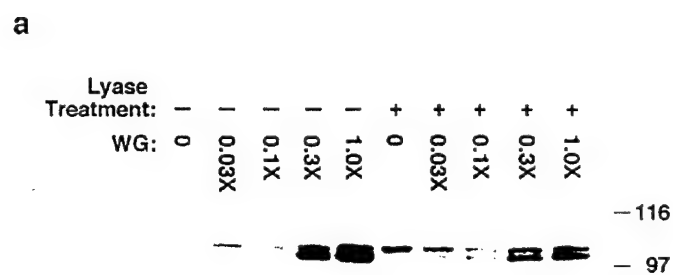


Figure 4b

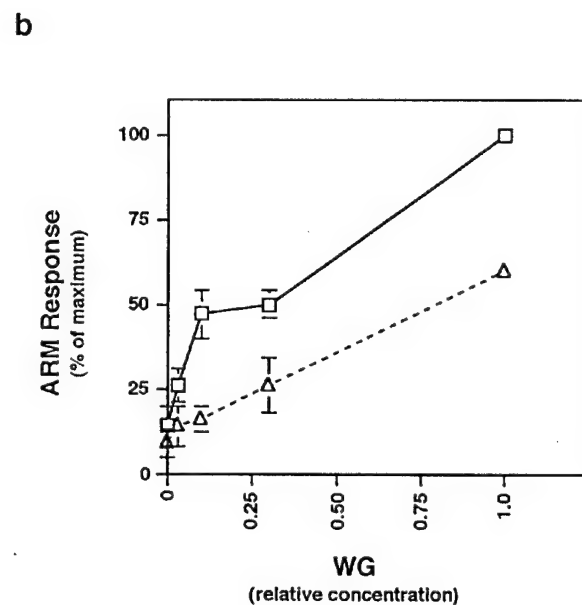




Figure 5

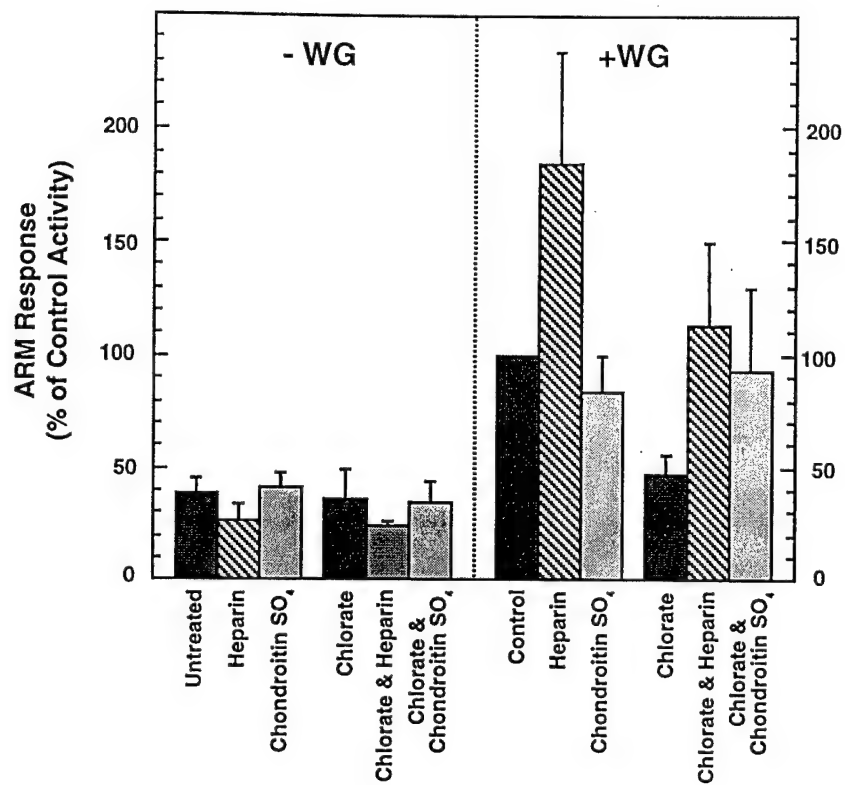


Figure 6 a

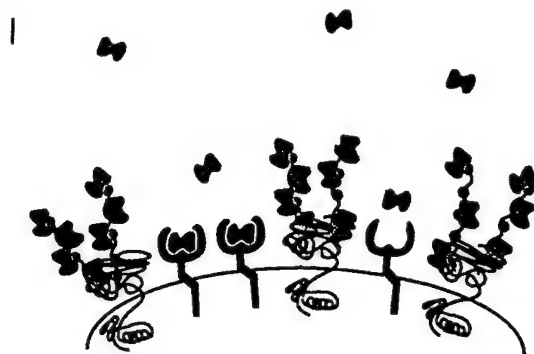
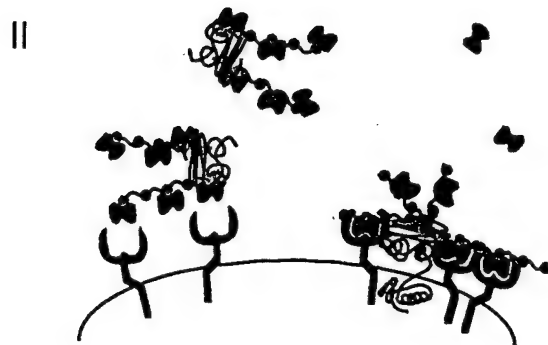


Figure 6 b



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## Post-Translational Modification and Secretion of *wingless* Protein

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Running title: Modification and Secretion of WG protein

Key words: *wingless*, *Drosophila*, WNT genes, *porcupine*, secretion, glycosylation

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**Background:** The *Drosophila* gene *wingless* and its murine counterpart *Wnt-1* encode secreted proteins which act as short range signals during development. We have analyzed some of the steps required for the synthesis and secretion of active *wingless* protein (WG). Here, we report that *porcupine*, a gene thought to function upstream in the *wingless* pathway, is required for both modification and secretion of WG.

**Results:** We show that that three distinct electrophoretic forms of WG can be detected both in vivo and in transgenic S2 cells expressing *wingless*. Two of the isoforms contain N-linked high mannose oligosaccharides. In *porcupine*<sup>-</sup> animals WG glycosylation is inhibited, and there is an accumulation of the intermediate glycoform. By examining WG expression and secretion in specific neuronal cells in the optic lobes, we have found that in WG is translocated along axon-like projections and secreted within the target regions of the developing ganglia of the optic lobe. In *porcupine*<sup>-</sup> animals, WG secretion is blocked very early. The protein accumulates in the cell bodies and is not translocated down the axons. Secretion of other growth factor-like proteins, such as *decapentaplegic* protein is not affected. Surprisingly, WG secretion can also be inhibited very late in the secretory pathway. In animals homozygous for a temperature sensitive allele of *wg*, *wg*<sup>IL114ts</sup>, the mutant protein is still translocated down the axons, but accumulates in the region near the cell membrane.

**Conclusions:** Our results demonstrate that WG is post-translationally modified by the addition of high mannose glycans. Loss of *porcupine* activity disrupts both post-translational modification and translocation of WG. Our findings that WG secretion can be blocked both early and late in the secretory pathway, without affecting secretion of other proteins, lead us to propose that WG secretion may be regulated rather than constitutive.

## Background

The *Drosophila* gene *wingless* is a member of the WNT gene family. The WNT genes have been implicated in a variety of developmental processes, and many were first identified as proto-oncogenes [for recent reviews see 1-3]. WNT proteins are highly conserved and likely function as secreted glycoproteins. They all contain a signal sequence, 23 conserved cysteine residues, and multiple potential N-linked glycosylation sites. *wingless* and its murine ortholog *Wnt-1*, are the best characterized members of the family. Both are required for development of the embryonic CNS. *Wnt-1* null mutations result in the loss of the midbrain and hindbrain structures in mice [4-5]; conversely ectopic *Wnt-1* expression induces neuronal axis duplication in *Xenopus* [6]. *wingless* is required for development of specific embryonic neuroblasts [7] and the adult optic lobes [8]. It also functions during a myriad of other developmental events, including epidermal patterning, midgut patterning, and limb formation. Genetic and immunocytochemical studies have shown that *wingless* protein (WG) can be secreted several cell diameters away from *wg*-expressing cells [9-11]. WG and other WNT proteins are relatively large compared to many secreted growth factors like EGF and TGF, and little is known about how they are transmitted from one cell to another in vivo.

It is intriguing that the post-translational modification, secretion and activity of Wnt-1 in vitro is highly dependent on the cell type in which it is expressed. For example, when Wnt-1 is expressed in C57MG cells [12], two Wnt-1 glycoforms are secreted [13] and localized to the cell surface, extracellular matrix (ECM) and medium. Both the conditioned medium and ECM fractions contain biologically active Wnt-1, as measured by their ability to induce transformation of mammary epithelial cells in co-culture experiments. In contrast, when Wnt-1 is expressed in many other cell lines [14-15], extracellular Wnt-1 can be detected only on the cell surface and ECM. The majority of Wnt-1 protein remains trapped in the secretory pathway, and appears to be only partially glycosylated. Furthermore, the extracellular Wnt-1 that is present is inactive: ECM fractions from these cell lines do not

transform mammary epithelial cells [12, 16]. These observations suggest that the synthesis and secretion of active Wnt-1 protein requires cell-type specific factor(s).

What about WG? *wg* expression has been studied only in transgenic S2 cells. In these cells WG is secreted and extracellular WG can be found on the cell surface, ECM and medium [17-19]. The secreted WG is biologically active, as measured by the ability to regulate turnover of *armadillo* protein in clone-8 cells [20]. It is not known whether WG, like Wnt-1, is glycosylated. van den Heuvel et al. [18] have reported that WG contains one potential glycosylation site which is conserved between WG and Wnt-1; however, glycosylation of WG has not been examined directly.

Genetic studies in *Drosophila* have identified at least one gene in the signaling pathway that plays an important role in WG secretion. Most of the extant genes act downstream in responding cells, but epistasis studies have shown that *porcupine* (*porc*) functions upstream of WG [21-23]. In *wild type* embryos WG protein can be detected several cell diameters away from *wg*-expressing cells. In *porc*<sup>-</sup> embryos, WG protein is found only within those cells that transcribe *wg* RNA, suggesting that *porc* is required for the secretion or transport of WG [18, 22-25]. However, it is also possible that *porc* is needed for stabilizing or localizing extracellular WG. In *porc*<sup>-</sup> embryos, extracellular WG may be rapidly degraded or simply diffuse away.

We are interested in the steps involved in both post-translational modification and secretion of WG. Here, we show that WG is modified by the addition of multiple N-linked oligomannose carbohydrates. We also describe the subcellular localization of WG in a cluster of large neuronal-like cells in the optic lobes. We show that WG is translocated along axon-like bundles into the developing ganglia. In *porc*<sup>-</sup> animals the translocation step is blocked: WG never travels from the ER to the cell surface. Surprisingly, loss of *porc* also effects the glycosylation of WG. We propose two models to explain the role of *porc* in the *wg* signalling pathway.

## Results

### Multiple forms of WG protein are expressed in vitro and in vivo

The post-translational processing of WG was examined in vitro using a transgenic Schneider 2 cell line which expresses WG under the control of a heat shock promoter (S2 HSWG(+)) cells [17]). Western analyses of whole cell extracts showed that three distinct forms of intracellular WG can be detected in S2 HSWG(+) cells (Fig. 1); while control S2 HSWG(-) cells did not express detectable amounts of WG. The three bands migrated with apparent molecular masses of 52, 55, and 57 kDa and hereafter are referred to as forms I, II, and III, respectively. A faint fourth band (59 kDa, form IV) can also be detected occasionally. Forms I, II, and III were also present in conditioned medium from S2 HSWG(+) cells (Fig. 1). Previous molecular analyses of the *wingless* gene indicate that it encodes only one polypeptide. A single 3 kb transcript was identified by Northern analysis; the *wg* cDNA has a 1419 nucleotide long open reading frame; and the deduced protein is 486 amino acids long with a predicted molecular mass of 52.7 kDa [26, 27]. Therefore, the fact that multiple forms of the WG protein were detected suggests that it is post-translationally modified in S2 cells.

To determine whether post-translational modifications also occur in vivo, we also carried out Western analysis on *HS-wg* embryos. WG protein is expressed at such low levels in wild type embryos that we are not able to reliably detect expression by Western analysis. Therefore, we used a *HS-wg* strain [28]. *HS-wg/TM3Sb* embryos (0-8 hours after egg lay) were collected, then heat shocked at 37°C for 1 hour and recovered for 1 hour at 25°C. As shown in Fig. 1, whole cell extracts from *HS-wg* embryos contained two forms of WG. These bands co-migrated on the gel with the slower migrating forms II and III found in S2 HSWG(+) cells. Very low levels of form I could be detected only rarely, suggesting that form I is rapidly processed into II and III. The large majority of embryonic WG protein was present as form III.

### WG is modified by N-linked glycosylation

Brown et al. [14] have shown previously that Wnt-1 is modified by N-linked glycosylation. In addition, Rijsewijk et al. [27] compared the WG amino acid sequence to Wnt-1 and found one conserved potential glycosylation site in WG at Asn 414. To test whether WG is in fact glycosylated, we examined the effects of tunicamycin treatment (an inhibitor of N-linked glycosylation) on WG processing. As shown in Fig. 2A, S2HSWG(+) cells grown in the presence of tunicamycin produced only form I, the 52 kDa species. This demonstrates that in vitro WG, like Wnt-1, is an N-linked glycosylated protein. Furthermore both forms II and III contain N-linked oligosaccharides.

The presence of two WG glycoforms could be due to (1) multiple glycosylation events; (2) processing of the mannose glycans into hybrid or complex oligosaccharides; or (3) additional modification to the protein backbone (e.g. phosphorylation or proteolysis). To distinguish between these possibilities we tested all three forms for sensitivity to Peptide: N-glycosidase F (PNGase F) and Endo-*B-N*-acetylglucosaminidase H (Endo H) cleavage. PNGase F cleaves the B-aspartyl-glycosylamine bond between the Asn and any oligosaccharide (including high mannose, hybrid, or complex oligosaccharides). After immunoprecipitation from whole cell lysates and then treatment with Peptide: N-glycosidase F (PNGase F), WG migrated as a single band (Fig. 3B), with the same mobility as form I. We conclude that the differences between forms II and III are contained within the carbohydrate moieties and are not due to processing of the core protein. Endo H is an endoglycosidase which cleaves between the GlcNAc residues of the glycan unit linked to the Asn. The specificity is such that oligomannose glycans are cleaved, but complex glycans and most hybrid glycans are not. Following Endo H digestion only form I can be detected (Fig. 2); both forms II and III were cleaved by endoH. WG protein also bound to the lectin from *Galanthus nivalis*, which has a high specificity for non-reducing terminal  $\alpha$ 1-3 mannose residues, but not *Lens culinaris* agglutinin which recognizes branched mannose groups with an  $\alpha$ 1-6 fucose (Reichsman and Cumberledge, unpublished results). Taken together, these



results are consistent with the model whereby form I is unglycosylated and forms II and III contain high mannose type oligosaccharides.

If WG is glycosylated at multiple sites, where are they located? We used the GCG sequence analysis program (Genetics Computer Group, Madison, WI) to search the amino acid sequence for the N-X-S/T motif and found four potential glycosylation sites (Fig. 3). We compared the WG sequence to eighteen other Wnt sequences (Fig. 3) and found that two of the four WG potential glycosylation sites are highly conserved. If all four sites are utilized we would expect to see 5 bands by Western analysis; the fact that we detect only 3 suggests that not all of the sites are actually glycosylated.

The first site (NIT at residues 37-39) is followed immediately by a P at amino acid 40, and therefore not likely to be glycosylated [29]; nor is not present in other Wnt proteins [30]. The second site (NCS at amino acids 103-106) is highly conserved. Of the 19 Wnt sequences we examined, 12 are NCS/T, 5 are NCP and one is NCN. The invariant C residue is known to play an important functional role in WG activity. When C104 in WG is replaced with an S, (e.g. *wg<sup>IL114ts</sup>*) the resulting protein is temperature sensitive for both secretion and activity [18, 31]. If C104 normally participates in a disulfide bond, the putative glycosylation site may not be available for modification.

The third and fourth sites appear to be the best candidates for glycosylation. The third site is found four amino acids downstream from the second (NFS at residues 108-110). It is present only in WG and Wnt-5a and 5b. The last potential glycosylation site (NET at residues 414-416) is present in 16 of the 19 Wnt proteins [30]. Mason et al. [13] demonstrated previously that this NET is glycosylated in the mouse Wnt-1 protein (N357). Changing N357 to a Q alters the glycosylation pattern of Wnt-1 and confers a temperature sensitive phenotype on the cells. It is interesting to note that the mutant protein is also hyper-secreted [13]. More extensive mutational and structural analyses will be required to demonstrate the actual WG glycosylation sites utilized in vivo.

### Loss of *porc* affects WG glycosylation

Several lines of evidence suggest that *porc* may be required for the secretion of active WG protein. Clonal analyses and epistasis experiments have shown that *porc* is needed in *wg*-expressing cells and that it acts upstream in the pathway [9, 22]. In addition, immunocytochemical studies [18] have shown that extracellular WG protein cannot be detected in *porc*<sup>-</sup> animals. These observations led us to ask whether *porc* is needed for post-translational modification of WG. We examined the glycosylation of WG in both control and *porc*<sup>-</sup> larvae. To examine WG expression in control larvae, we picked 26 single third instar *HS-wg* males, prepared whole larval extracts, and then analyzed individual larvae by Western blotting (Fig. 4). In all 26 *HS-wg* larvae examined, the vast majority of WG migrated as form III. Next, we examined 56 males from the cross *HS-wg/TM6Tb; +/Y* × *+/+; porc<sup>PB16</sup>/FM7*. Since *porc* is located on the X chromosome, half of the *HS-wg* males will be *porc<sup>PB16</sup>* and half will be *porc*<sup>+</sup>. Although *porc<sup>PB16</sup>* is a null allele, *porc*<sup>-</sup> animals have a supply of maternally-provided *porc* gene product and do not die until the pupal stage [25]. However, by late third instar, these stores have been depleted in *porc<sup>PB16</sup>* males and WG protein is no longer secreted (see Fig. 5 below). In approximately half, 31 of 56, males examined (the putative *porc*<sup>+</sup> males) the expression pattern was normal: high levels of form III with little or no detectable form II (Fig. 4). In the remaining half, the *porc*<sup>-</sup> males, WG expression was altered two ways. First, there was a significant increase in the total amount of WG detected in each larvae, indicating that when secretion is blocked there is a concomitant accumulation of intracellular WG. Second, equivalent amounts of form II and III were expressed in *porc*<sup>-</sup> animals. The increase in form II suggests that loss of *porc* inhibits WG glycosylation. Nonetheless, some form III was still present. We interpret this to mean that *porc* is not absolutely required for glycosylation, but rather, that the accumulation of form II may be an indirect consequence of the loss of *porc*.

### *porc* is required for translocation of WG along the secretory machinery

Previous studies using immunocytochemical techniques to examine WG distribution in *porc*<sup>-</sup> animals were carried out using embryonic or larval epithelium, tissues where the cells are quite small. Therefore, it was not possible to examine the subcellular localization of WG. We overcame this limitation by studying WG expression in the large lamina precursor cells found in the larval optic lobe. Some of these cells, which extend long axon-like projections into the lamina target region, express WG [32]. Because of their size and neuronal-like morphology, they provide a unique system in which to study the subcellular distribution of WG protein in both wild type and mutant animals.

When wild type CNS tissue were stained with anti-WG antibody, WG protein could be detected in two clusters of cells at the surface of the optic lobe (Fig. 5A). These cells correspond to the  $\beta$ -gal expressing cells described by Kaphingst and Kunes [8] using an *wg-lacZ* enhancer trap strain (compare Fig. 5A and B), and are the termini of the lamina target region. Note that the anti-WG staining was diffuse compared to the anti- $\beta$ -gal staining.

In addition, WG protein also accumulates internally, in a crescent-shaped structure within the optic lobes (Fig. 5A). Double staining with anti-WG antibody and Mab22C10 revealed that this crescent structure is the developing lamina neuropil, which is innervated by axons from the photoreceptor neurons in eye disc. It is important to note that the WG protein is localized within the region where synaptic connections are forming between the central neurons of the brain and retinal neurons. How does the WG protein reach the lamina target region? Close inspection of the  $\beta$ -gal positive cells near the surface reveals that they send long axon-like projections to the target neuropil (Fig 5C,D). The WG protein appears to be translocated from the cell bodies near the lobe surface, down the axon-like extensions, into the developing lamina ganglion.

We then examined the subcellular distribution of WG in *wg<sup>IL114ts</sup>* larvae (Fig 5C,D), where WG secretion is blocked. *wg<sup>IL114ts</sup>* is a conditional lethal allele. In vitro studies by van den Hueval et al [31] have demonstrated that *wg<sup>IL114ts</sup>* protein is not secreted at the restrictive temperature. On vivo, when raised at 16.5°C, *wg<sup>IL114ts</sup>* animals are viable. But

when raised at 25°C, *wg<sup>IL114ts</sup>* embryos have a segment polarity phenotype, no extracellular WG protein can be detected, and the animals die [11, 18, 31]. Antibody staining of fixed optic lobe tissue showed that WG protein still accumulated in both the cell bodies and in the lamina target region (Fig. 5E) at 25°C. In fact, the staining pattern in the *wg<sup>IL114ts</sup>* mutant animals was more intense, more discreet and less diffuse than what we observed for wild type larvae. This is consistent with the idea that WG accumulates intracellularly when secretion is blocked. It is striking that the protein was still found in the developing lamina. Even though it was not secreted, *wg<sup>IL114ts</sup>* protein was still translocated along the secretory pathway to the lamina neuropil.

Next, we compared this staining pattern with WG expression in *porc*<sup>-</sup> animals. To examine WG expression in *porc*<sup>-</sup> animals, we dissected optic lobes from late third instar *porc<sup>PB16</sup>* males, then fixed and stained with anti-WG antibody. The intracellular distribution of WG was markedly different in *porc*<sup>-</sup> larvae. As shown in Fig. 5E and F, WG was found only within the cell bodies in *porc*<sup>-</sup> animals. It no longer traveled to the lamina target structure. The WG protein was apparently trapped at some early step(s) in the secretory pathway, and was not translocated out to the nerve terminus. Stronger anti-WG staining was seen consistently in the cell bodies of *porc*<sup>-</sup> animals as compared to wild type, suggesting that when secretion is blocked, WG accumulates within the cell. Our results showing that in *porc*<sup>-</sup> animals, WG glycosylation is inhibited and translocation is blocked, strongly support the model whereby *porc* is required for secretion of WG.

Genetic analysis of the *porc*- phenotype [14] had suggested that *porc* is not required for secretion of all growth factors. We tested this directly by examining *decapentaplegic* (*dpp*) expression in the optic lobes of *wild-type* and *porc*<sup>-</sup> animals. Adjacent to the WG-expressing cells are two clusters of cells that secrete *dpp* protein (DPP) [8, 32]. To identify the DPP expressing cells, we examined DPP expression both in wild type larvae, and in animals genetically engineered to express high levels of DPP (*GAL4-dpp; UAS-dpp*) (Fig. 5G, H). Like WG, DPP was expressed in two clusters near the dorsoventral boundary. In the wild-

type animals DPP expression is very diffuse and somewhat faint. DPP expression in the *GAL4-dpp; UAS-dpp* was somewhat stronger, but again the DPP protein was quite diffuse. This staining pattern is probably characteristic of a secreted protein. Next, we examined DPP expression in *porc*<sup>-</sup> larvae (Fig. 5I). We found identical anti-DPP staining patterns in both *wild-type* and *porc*<sup>-</sup> larvae indicating that DPP is still secreted in *porc*<sup>PB16</sup> males. These results suggest that *porc* encodes a factor needed specifically for the translocation of WG from the ER to the plasma membrane for secretion.

## Discussion

We have shown that WG is post-translationally modified. Three distinct electrophoretic isoforms (I, II, and III, with an apparent molecular mass of 52, 55 and 57 kDa respectively) can be detected in both transgenic S2 cells and *in vivo*. Previous work by van den Heuvel et al. [18] had reported only one form of WG in *wg*-expressing S2 cells. This difference might be explained by the specificities of the different anti-WG antibodies used in the two studies. The anti-WG antibody used by van den Heuvel et al. [18] cross-reacted with a 52 kDa protein present in both control and *wg*-expressing S2 cells; this cross-reactivity may have masked the other WG isoforms present.

Our experiments using tunicamycin treatment and Endo H digestion indicate that Form II and III are glycoproteins with N-linked carbohydrates, most likely of the high mannose types. This is consistent with previous studies on glycosylation in insects. Although most insect cells are able to carry out the addition of high mannose oligosaccharides, many lack some of the key enzymes needed to process the mannose groups into the hybrid and complex oligosaccharides found in vertebrates [33-36]. Furthermore, complex type oligosaccharides have not been documented in arthropod glycoproteins. It is not known whether the murine Wnt-1 glycans are of the high mannose, hybrid, or complex type.

Glycosylation may be a feature common to all WNT proteins. Burrus and McMahon [37] have examined the expression of six other murine Wnt proteins in COS cells and found that all are glycosylated. The functional significance of N-linked glycosylation of WNT proteins is not known. In other systems glycosylation can regulate a variety of properties, including protein folding, sorting, and extracellular stability of proteins. Our results show that glycosylation is not requisite for WG secretion, as the 52 kDa non-glycosylated isoform can be detected in conditioned medium from S2HSWG(+) cells (Fig. 1). This is supported by work from Mason et al. [13] showing that mutant Wnt-1 protein lacking all four glycosylation sites is still secreted. Glycosylation does alter at least one biochemical property

of WG: form III binds with higher affinity to heparin than form II [19]. This is interesting because glycosaminoglycans are thought to play a functional role in WG signaling.

Using immunocytochemical techniques to follow WG expression in the optic lobes, we have found that *porc* is required for the translocation of WG out to the cell surface. In addition, loss of *porc* activity results in the accumulation of glycoform II. Perrimon and co-workers [38] have demonstrated recently that *porc* encodes a multi-pass transmembrane protein localized within the secretory network. It will be interesting to determine if secretion of Wnt-1 also requires a vertebrate *porc* counterpart. Perhaps the variability in Wnt-1 secretion in different cell types can be explained by the presence or absence of *porc* activity.

How does *porc* affect processing events in the ER and secretion of WG? Several events must take place before WG can be secreted. As it is being synthesized, WG must first be translocated into the ER where the nascent polypeptide chain is glycosylated and folded. Then the protein is transported from the ER to the Golgi network, and ultimately shuttled to the cell surface for secretion. From the present information, we propose two possible models for *porc* action. In the first model the *porc* gene product is proposed to function as a molecular chaperone, mediating WG folding. Folding, glycosylation, and disulfide bond formation are all interdependent processes, and misfolded glycoproteins are known to accumulate in the ER. In addition, misfolding may interfere indirectly with normal glycosylation events. There is precedence for this model: the ER transmembrane chaperone, calnexin, is known to bind to secretory glycoproteins and promote folding [39].

In the second model, *porc* functions as a component of the secretory machinery, directing WG to the cell surface. For example, *porc* may be required for translocation of WG into the ER, targeting of WG to the correct vesicles, or vesicle shuttling. The accumulation of WG processing intermediates could be an indirect consequence of blocking the WG secretory path. The fact that *porc* is required for secretion of WG, but not DPP protein, raises the possibility that WG is not transported through the constitutive secretory pathway. The behavior of the WGIL114ts protein is also consistent with this idea. At the restrictive

temperature, the WGIL114ts protein is found near the cell surface but is not secreted, again suggesting that the later step(s) in WG secretion may require specialized secretory machinery. The two models are quite speculative and further information is needed before they can be rigorously evaluated. Analysis of the subcellular localization of the *porc* protein may help to differentiate between the possibilities, since many chaparones and pore proteins are found predominately in the ER; while proteins involved in vesicle fusion and trafficking would also be found in the Golgi and secretory vesicles.



## Materials and Methods

### *Cell Culture*

S2HSWG(+), WG-expressing cells contain the *wingless* cDNA under the control of the heat shock promoter. S2HSWG(-) control cells have the same construct in the anti-sense orientation [17]. S2 cells were cultured in Schneider's Drosophila Medium (Gibco) supplemented with 10% FCS (Gibco) and penicillin/streptomycin (Gibco) at 25°C.

### *Drosophila Strains and Growth conditions*

All flies were raised on standard cornmeal medium at 25°C unless noted. The *porc<sup>PB16</sup>* mutation was obtained from Norbert Perrimon [24]; *wg<sup>IL114 386</sup>* from Amy Besjovec [11, 18, 31]; HS-*wg/TM3Sb* flies from Roel Nusse [28] and *wg-lacZ 17en*(or 11en) from Judy Kassis [39]. *wg<sup>IL114 386</sup>* homozygous larvae were identified from the cross *wg<sup>IL114 386</sup>/T(2;3)CyTb* x *wg<sup>IL114 386</sup>/T(2;3)CyTb* by scoring for non-Tubby larvae. *wg<sup>IL114 386</sup>/wg-lacZ* larvae were obtained from the cross *wg<sup>IL114 386</sup>/T(2;3)CyTb* x *wg-lacZ 17en/T(2;3)CyTb*. *wg* activity was inactivated by shifting homozygous *wg<sup>IL114 386</sup>* larvae or heterozygous *wg<sup>IL114 386</sup>/wg-lacZ 17en* larvae from 16.5°C to 25°C at L2, the animals were then allowed to develop until wandering third instar. *Canton S* and *Df(1)w 67(1.23y)* flies were used as control strains.

### *Preparation of WG protein*

Whole cell extracts were prepared from S2HSWG(+) cell which had been heatshock at 37°C for 1 hour and then recovered for 1 hour at 25°C to induce WG expression. Cells were collected, washed in PBS, and lysed by boiling in 1X SDS sample buffer with 5%  $\beta$ -mercaptoethanol and bromophenol blue dye. To prepare WG-conditioned medium, S2HSWG(+) cells were grown to a density of  $6-8 \times 10^6$  cells/ml. The cells were then heat shocked for 1 hour at 37°C, washed twice, and allowed to recover in serum-free M3 medium at 24°C for 2-3 hours. The conditioned medium was separated from cells by centrifugation at

2,000g for 5 minutes, cleared by centrifugation at 20,000g for 15 minutes at 4°C, and concentrated in UF-30 spin filters (Integrated Separation Systems).

Whole embryonic extracts were prepared from embryos collected from *HS-wg/TM3Sb* flies. Embryos (0-8 hours after egg lay) were heatshocked for 1 hour at 37°C and recovered for 1 hour at 25°C, then collected, washed, dechorionated in 50% bleach for 2 minutes, and lysed in 50 mM Tris (6.8), 2% SDS using an eppendorf pestle. Glass beads were added and the sample was vortexed then heated to 95°C for 2 minutes. The lysate was removed and the protein concentration was measured. 5%  $\beta$ -mercaptoethanol and 0.1% bromophenol blue was added and samples (100  $\mu$ g of total protein per sample) were analyzed by immunoblotting. Larval extracts were prepared from non-Tubby male third instar larvae from the cross *HS-wg/TM6Tb* x *y w f porc<sup>PB16</sup>/FM7*. Individual larvae were washed in PBS, heatshock for 1 hour at 37°C, and recovered for 1 hour at 25°C. 2X SDS sample buffer (50 mM Tris (6.8), 2% SDS, 0.5 %  $\beta$ -mercaptoethanol, 0.1 % bromophenol blue, 1mM PMSF) was added to each larvae, and the tissue was lysed by grinding with an eppendorf pestle, then boiled for 2 minutes.

### *Immunoblotting*

Protein samples were subjected to 10% SDS-PAGE, and then transferred to nitrocellulose (Schleicher and Schuell). The membranes were blocked with 3% milk PBT (PBS, 0.05% Tween 20), probed with rabbit anti-WG antibody (1:8000 or 1:15,000 ) and secondary goat anti-rabbit-HRP antibody (1:3000, Biorad). The membranes were washed and antibody binding was detected using the ECL chemiluminescence substrate according to manufacture's protocol (Amersham or Super Signal, Pierce).

*Preparation of WG antibody*

Full length WG protein fused in frame with six histidine codons at the amino end was expressed in *E. coli* and purified by metal column chromatography using the Novagen pET-His tag system. The purified protein was mixed with Freund's adjuvant and used for injection in rabbits to produce a rabbit anti-WG polyclonal antibody.

*Tunicamycin treatment*

S2HSWG(+) cells were pre-incubated for 2 hrs in DMSO or 5  $\mu$ g/ml tunicamycin (10 mg/ml in DMSO) heatshocked 1 hour, and recovered 1 hour. Whole cell extracts from an equal numbers of treated and untreated cells were electrophoresed on a 10% gel and analyzed by Western blotting.

*Preparation of Fab<sub>2</sub> fragment for immunoprecipitation*

Both WG and the IgG heavy chain run at the same molecular mass (55 kDa) on an SDS denaturing gel. To differentiate between antibody and antigen during immunoprecipitation experiments we used biotinylated rabbit anti-WG F(ab)<sub>2</sub> fragment (25 kDa) followed by avidin conjugated agarose for all immunoprecipitations. F(ab)<sub>2</sub> fragment was generated from whole rabbit anti-WG antibody by digesting with 20  $\mu$ g pepsin (Sigma) per mg of antibody in 100 mM sodium citrate (3.5) for 6 hours at 37°C. The reaction was stopped with 1/10 volume of 3.0 M Tris (8.0) and the sample was dialyzed against 0.1 M sodium borate (8.8). To biotinylate the F(ab)<sub>2</sub>, 250  $\mu$ g N-hydroxysuccinimide ester (10 mg/ml in dimethyl sulfoxide) was added per mg of antibody and the solution was incubated at room temperature for 4 hours. The reaction was stopped by adding 20  $\mu$ l 1M NH<sub>4</sub>Cl per 250  $\mu$ g ester and incubating at room temperature for 10 minutes. Free biotin was removed by exhaustive dialysis against PBS. Avidin agarose beads (Pierce) were used to immunoprecipitate the F(ab)<sub>2</sub>-protein complexes.

*Glycosidase digestion*

Whole cell extracts from S2HSWG(+) cells were lysed in RIPA buffer, and WG was immunoprecipitated using an excess of F(ab)<sub>2</sub>. Protein-F(ab)<sub>2</sub> complexes were captured by avidin agarose beads, washed 3X in RIPA buffer. The avidin bead complex was washed 3X with RIPA. For PNGase F digestion, the immunoprecipitates were boiled 10 minutes. in 5X denaturing buffer (2.5% SDS, 5%  $\beta$ -mercaptoethanol), and diluted to 1X denaturing buffer with water. 1/10 volume of 10X G7 buffer (0.5 M sodium phosphate, (pH 7.5 ), 1/10 volume 10 % NP-40, and 2.5 mU or 0 mU (control) of PNGase F (New England Biolabs) were added and the samples were incubated at 37°C for 1 hour. The reaction was stopped by the addition of an equal amount of 2X SDS, the sample was boiled 5 minutes., electrophoresed and analyzed by Western blotting as described. For Endo H digestion, the immunoprecipitates were resuspended in 50  $\mu$ l of 50 mM citrate (5.5), 0.1% SDS buffer, heated to 95°C for 2 minutes. and then cooled. 2 mU or 0 mU of Endo H (Boehringer Mannheim) were added and incubated at 37°C for 3 hours. An equal volume of 2X SDS sample buffer was added and samples were electrophoresed on a 10% SDS gel, transferred to nitrocellulose, and analyzed by Western blotting as above.

*Immunocytochemistry*

For CNS dissections, wandering third instar larvae were cut in half and inverted inside out in PBS (pH 7.4) to expose the CNS, and extraneous tissue was removed. CNS were fixed in 4% formaldehyde (diluted in PBS) for 45 minutes at the room temperature. After several washes in PBSBT (1X PBS; 1% BSA; 0.1% Triton X-100), the samples were blocked for 3 hrs in PBSBT at the room temperature, incubated with primary antibodies diluted in PBSBT overnight at 4 °C with gentle shaking, washed with PBSBT for 4 times with a 30 minutes interval, incubated with secondary antibodies in PBSBT for 3 hours at the room temperature, followed by extensive washes with PBSBT, and then mounted in VECTASHIELD mounting medium (Vector Labs). Antibodies were used at the following dilutions: rabbit anti-WG 1:

500; rabbit anti- $\beta$ -galactosidase (Sigma) 1:1000; mouse anti- $\beta$ -galactosidase (Sigma) 1:1000; MAb22C10 [40] 1: 100; rabbit anti-DPP (provided by M. Hoffman) 1: 25; mouse anti-fas II (provided by C. Goodman) 1:10; FITC-conjugated donkey anti-rabbit (Jackson) 1: 1500; and Texas Red-conjugated goat anti-mouse (Jackson) 1:1000.

### *Confocal microscopy*

Specimens were viewed under a Bio-Rad MRC-600 confocal microscope with an argon laser. Fluorescein and rhodamine fluorescence were individually detected with the A.1 and A.2 filter blocks. Each image was obtained about 5-10 scans through the Kalman-averaged program in the Bio-Rad COMOS software. Image were analyzed using NIH image 1.58 and Adobe Photoshop 3.0.4 software.

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## Figure Legends

**Figure 1.** Western blot of WG isoforms expressed both in vitro and in vivo.

S2 HSWG(-) cells, S2 HSWG(+) cells, and *hs-wg/TM3Sb* embryos (2-11 hours after egg laying), were heatshocked 1hr at 37°C and allowed to recover at 25°C. Whole cell extracts and WG-conditioned medium were then prepared as described, subjected to 10% SDS-PAGE, transferred to nitrocellulose, and probed with rabbit anti-WG antibody.

**Figure 2** (A) Tunicamycin blocks post-translational modification of WG.

S2 HSWG(+) cells were pre-incubated for 2 hours in the presence or absence of tunicamycin (an inhibitor of N-linked glycosylation), heatshocked 1 hour and allowed to recover for 1 hour. Whole cell extracts were prepared and analyzed by Western analysis. (B) PNGase F cleaves glycoforms forms II and III.

WG was immunoprecipitated from S2 HSWG(+) whole cell extracts, then treated with and without Peptide:N-glycosidase F (PNGaseF), which removes all oligosaccharides, including high mannose, hybrid or complex, from Asn residues. After digestion samples were analyzed by Western blotting.

(C) Glycoforms II and III are Endo H sensitive. WG was immunoprecipitated from S2 HSWG(+) cells, then treated with and without Endonuclease H (Endo H) which removes oligomannoses from Asn residues. After digestion samples were analyzed by Western blotting.

**Figure 3** Partial sequence alignment of 19 Wnt proteins.

The four potential Asn-linked glycosylation sites found in the WG amino acid sequence are aligned with the corresponding sequences found in other Wnt proteins. Sequence alignments are taken from Sidow, 1992. The A-X-S/T consensus sequences are shown in bold.

Sequences were obtained from the Swiss-Prot Protein Sequence Database. Accession

numbers are: *Drosophila* Wnt-1 P09615; mouse Wnt-1 P04426; axolotl Wnt-1 P21551; *C. elegans* Wnt-1 P34888; *xenopus* Wnt-1 P10108; zebrafish Wnt-1 P24257; *Drosophila* Wnt-2 P28465; mouse Wnt-2 P21552; *C. elegans* Wnt-2 P34889; mouse Wnt-3 P17553; mouse Wnt-3a P27467; mouse Wnt-4 P22724; mouse Wnt-5a P22725; mouse Wnt-5b P22726; mouse Wnt-6 P22727; mouse Wnt-7a P24383; mouse Wnt-7b P28047; *xenopus* Wnt-8 P28026; mouse Wnt-11 P48615.

**Figure 4** WG glycosylation is effected by loss of *porcupine*.

Western blot of whole larval extracts prepared from individual wandering third instar larva.

Open circles: *HS-wg*; *porc<sup>PB16</sup>* and *HS-wg*; *FM7* males from the cross *HS-wg/TM6Tb*; *+Y* x *+/+*; *porc<sup>PB16</sup>/FM7*; closed circles: control *HS-wg / TM6Tb* males.

**Figure 5** Localization of WG protein in lamina precursor cells of the optic lobes.

All images are of late third instar larval optic lobes. Optical sections taken by confocal microscopy. Lateral view; dorsal up; posterior to the left. (A) Anti-WG antibody staining (green) in wild type animals. WG is localized in two clusters of cells near the surface. These cells straddle the dorsoventral midline (Arrow). In addition, WG protein can be found deeper, within the developing lamina target region. Photoreceptor axons (visualized by MAb 22C10 antibody staining; red) also innervate the target region. (B)  $\beta$ -gal staining (green) in *wg-lacZ* animals. The two  $\beta$ -gal domains are localized on opposite sides of the dorsoventral midline. The lamina is visualized by anti-fas II antibody staining (red). (C) WG (green) can still accumulate in the lamina target region in *wg<sup>IL114ts</sup>/wg-lacZ* larvae. Although *WG<sup>IL114ts</sup>* protein is not secreted, it can be detected both in the clusters of cell bodies near the surface, and deeper in the lamina target region. Note that the  $\beta$ -gal positive cells extend projections into the lamina target region (indicated by arrow heads). (D) Higher magnification of C. WG can be found along the axonal-like projections traveling from the cell bodies to the target neuropil. A thick arrow marks the dorsoventral midline of the larval optic lobes. (E)

Translocation of WG (green) is blocked in *porc<sup>PB16</sup>* larvae. WG is confined to the two domains which straddle dorsoventral midline. No staining is found in the lamina target region. The lamina is visualized by a-fas II antibody staining (red). (F) Anti-WG antibody staining (green) and anti-  $\beta$ -gal antibody staining (red) co-localize (yellow) in *wg-lacZ*; *porc<sup>PB16</sup>* animals. (G) Double staining with anti-DPP antibody (green) anti-fas II antibody staining (red). The two clusters of DPP positive cells are located adjacent to wg-expressing cells. Arrow marks dorsoventral midline. (H) Anti-DPP staining of wild type. (I) Anti-DPP staining of *porc<sup>PB16</sup>* animals. Scale bars are 20  $\mu$ m. Abbreviations: os, optic stalk; la, lamina; lo, lobulla; br, brain.

Figure 1

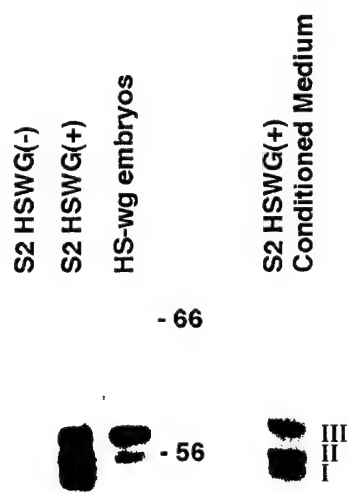


Figure 2

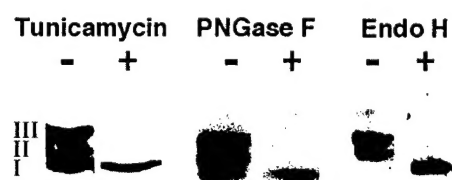


Figure 3

Drosophila Wnt-1 (WC)	WWGIAKVGEPNNT-----PIMYM	56	QHQFNRNRWNCSTRNFSR-GKNLFGKIV	120	ILGTHGRQCNETSLGVDG	422
mouse Wnt-1	WVGIVNIASNTNLL--TDSKSLQLVL	57	KWQFNRNRWNCPT---AP-GPHLFGKIV	117	TAGTAGRACNSSSPALDG	324
axolotl Wnt-1	WVGIVNVVTSTNLL--TDTKNVQLVL	56	KWQFNRNRWNCPT---TG-GDNIFGKIV	116	TSQTSGRVCNSSSLGLDG	323
C. elegans Wnt-1	SW-LALGLAANRF-----	33	QQFHKRRWNCCTL---ID-----PVT	95	ILGTHGRQCCTLAPNAIDD	326
xenopus Wnt-1	WVGIVNVASAGNVLPQSDARPVPLVL	57	KWHFNRNRWNCPT---GT-GNQVFGKII	117	TPGTGTRICNSTSLGLDG	325
zebrafish Wnt-1	WVGIVNVASSGNLL--TNSKNVQLVL	56	KWQFNRNRWNCPT---TH-SPNVFGKIV	116	THGTSGRTCNSSSPALDG	324
Drosophila Wnt-2	--SSF-----	24	QHQFGRHRWNCSE---VW-QRNVFAHVI	89	SQTSGRTCQRTGHGPQS	306
mouse Wnt-2	WWMRATGG-----	35	QHQFGRHRWNCNT---LDRDHSFGKIV	101	SLGTAGRVCNLTGRGMS	303
C. elegans Wnt-2	WWSVAQLSTALAGH-----	40	QRQFTGRHRWNCST---HY-STGMLGPIH	104	TLGTTEGRVCKRGSGGAG	314
mouse Wnt-3	WWSLALGQYTSLSA-----	39	QHQFGRHRWNCCTT---IDSLAIFGPVL	105	SFGTRDRTCNVTSHGIDG	309
mouse Wnt-3a	WWSLAVGPQYSSLS-----	56	QHQFGRHRWNCCTT---VNSLAIFGPVL	102	SFGTRDRTCNVSSHGIDG	306
mouse Wnt-4	--SSVGS-----	37	QYQFNRNRWNCST---LD-SLPVFGKV	102	VLGTRGRTCNKTSKAIDG	305
mouse Wnt-5a	WWSLGMNPNVQMSVYII-----	63	QYQFNRNRWNCST---VD-NTSVFGRVM	128	SLGTQGRCLCNKTSEGMDG	333
mouse Wnt-5b	WWSLAL-NPVQPEMFII-----	55	QHQFQRNRWNCST---VD-NTSVFGRVM	120	SLGTQGRCLCNKTSEGMDG	326
mouse Wnt-6	WWAVGSPVM-----	34	QHQFNRNRWNCSS---HS---KATGRVL	97	SPGTRGRACNSSAPDLG	318
mouse Wnt-7a	--SSVAL-----	32	QYQFNRGRWNCSA---LG-ERTVFGKEL	97	SVGTQGRACNKTAPQASG	303
mouse Wnt-7b	--SSVAL-----	32	QHQFNRGRWNCSA---LG-EKTVFGQEL	97	SVGTQGRCLCNRTSPGADG	303
Xenopus Wnt-8	SAWSVNNFLMT-----	32	KYQFAWRWNCPE---ST-LQLATHNGL	79	LQGTGRECLQSGKNLSQ	285
mouse Wnt-11	WALASKTPAALALNQTOHCK-----	56	RRAFADMNRWNCSSSIELYPNYLLDLERGT	108	SHGTQDRQCNCNKTNGSDS	308

Figure 4

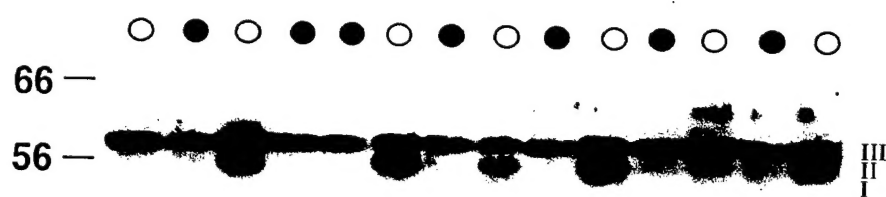


Figure 5

